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Essential Role for CAML in Follicular B Cell Survival and Homeostasis

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Abstract

Calcium-modulating cyclophilin ligand (CAML) is a ubiquitously expressed protein that is important during thymopoiesis. However, whether or not it serves a function in mature lymphocytes is unknown. Here, 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 antigen receptor stimulation and to B cell activating factor (BAFF), they displayed decreased survival and increased apoptosis following stimulation with LPS and IL4 *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.

Introduction

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, step-wise process of maturation during which cells express a defined series of cell surface markers that enable their identification (1). Hardy, *et al.*, identified six developmental stages in the bone marrow, designating them Fractions (Fr) A through F, while Loder, *et al*., further identified B cell developmental stages within the spleen. In this system, bone marrow Fr 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 B cell activating factor (BAFF) binding to and activating its highest-affinity cognate receptor, BAFF Receptor (BAFF-R). Deletion in mice of either BAFF or BAFF-R substantially reduces the populations of mature splenic B cells (3–5).

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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 (ER)(7). Although its mechanism of action is not yet deciphered, it is known to physically interact with several signaling molecules, including $p56^{lck}$ (8), the B cell-specific tumor necrosis factor receptor (TNFR) family member transmembrane activator and CAML interactor (TACI) (9), and the Epidermal Growth Factor Receptor (EGFR), 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 knock-out mice (designated *bCAML*−/−) and tamoxifen-inducible CAML knock-out 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 antibodies

MicroBeads specific for murine antigens CD8a, CD4, CD19, & goat anti-rat IgG and APC conjugated antibodies specific for murine CD43 were from Miltenyi Biotec. APC conjugated antibodies specific for CD23 were from Southern Biotech. FITC conjugated antibodies specific for CD23 were from eBioscience. APC conjugated antibodies specific for CD21 were from BioLegend. Tamoxifen, corn oil, & Lipopolysacharide from E. coli O55:B5 were from Sigma Aldrich. Unconjugated antibodies specific for CD16/CD32 (to block Fc receptors) & FITC conjugated antibodies specific for BP1, CD5, CD93, IgD, IgG1, BrdU and isotype controls, PE conjugated antibodies against B220, CD21, CD69, IgA, MHCII, & TLR4, PerCP-Cy5.5 conjugated antibodies against B220 & IgM, Cy5 conjugated AnnexinV, APC conjugated antibodies against B220 & CD19, and Propridium Iodide staining solution were from BD Pharmingen. CAML specific antibodies were previously described (8).

Mice

Conditional B cell knock-out mice, designated *bCAML*−/− *(CD19-Cre,*$CAML^{fl/fl}$), 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+CAMLfl/+ mice (data not shown). Tamoxifen-inducible CAML knock-out mice, designated *eCAML*−/− (*ESR-cre,*$CAML^{fl/fl}$) mice were generated by crossing ESR-cre mice (15) to CAML flox/flox mice, and sacrificed between 13 and 26 weeks. NOD/SCID mice were acquired from Jackson Laboratories and sacrificed between 20–32 weeks. 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, and bone marrow were isolated, made into single cell suspensions and stained with fluorescent dye-conjugated antibodies and CD16/CD32 (to prevent non-specific antibody binding). AnnexinV and PI staining was conducted as per

manufacturers' directions (BD Biosciences). Live lymphocytes were identified based on forward and side scatter or, as indicated, by negativity for PI and AnnexinV.

Purification of mature murine B cells

Splenocytes were cultured in RPMI with 10%FCS, β-mercaptoethanol, penicillin & streptomycin, & L-glutamine for 30 min at 37°C in tissue-culture treated 6-well plates to allow for adherence of macrophages and dendritic cells. Non-adherent cells were bound to CD4 & CD8 Microbeads (Miltenyi Biotec) to negatively select T cells. Transitional B cells were negatively selected based on CD93 expression using rat anti-CD93 abs and goat anti-rat IgG Microbeads. Remaining mature B splenocytes were cultured and stimulated under conditions described.

B Cell Receptor Stimulation in Culture

Total splenic B cell compartment was isolated, as previously described, and cultured at 1million cells/ml without stimulation or with 10ug/ml AffiniPure $F(ab')_2$ fragment (Jackson ImmunoResearch). Cells were stained at 0 and 12 hrs for expression of CD93, CD19, CD23, & CD69. Live cells were determined based on forward and side scatter and PI exclusion. Fo cells were CD19+, CD23+, CD93−. MZ cells were CD19+, CD23−, CD93−.

Lipopolysacharide and IL4 stimulation and culture

Purified murine mature B cells, consisting of both MZ and Fo B cells, were cultured at 37°C with 5%CO_2 in sterile conditions, and stimulated with LPS (15ug/ml) and IL-4 (30ng/ml) at 1 million cells/ml in 24 well plates. Fresh media containing LPS (15ug/ml) was added after 48 hours. Cells were removed from culture and stained with CD23, AnnexinV, and PI at the indicated time points. Numbers of MZ and Fo cells were determined via FACS and plotted as the percent of cells per ml present at each time compared to the 0 hr time-point. *bCAML* and *eCAML* mice data were the result of 2 separate experiments each. For comparison between separate experiments in *eCAML* mice, values were adjusted to the cell number of control samples within each experiment.

BAFF and IL4 stimulation and culture

Purified mature splenocytes were cultured at 1.5 million/ml in 100ul aliquots and stimulated with BAFF (1ug/ml), IL4 (50ng/ml), or no stimulation. Samples were taken at 0, 36, 66, & 96 hrs post-stimulation in triplicate. Live cells were identified by negative staining for annexinV and PI; Fo cells identified via CD23+ staining, while MZ cells were CD23−.

CFSE staining and stimulation

Lymphocytes were stained in CFSE (5uM) in PBS for 30 minutes at 37°C, then washed 3x in RPMI with 10%FCS. Cells were stimulated with LPS (15ug/ml) and IL-4 (30ng/ml) and cultured in media for the times described at an initial concentration of 1million cells/ml. Cells were recovered and resuspended in fresh media containing LPS (15ug/ml) after 24 hrs.

Tamoxifen and BrdU injections

Mice were injected IP with 1.5mg of sterile-filtered Tamoxifen in corn oil (10mg/ml, 150ml total per injection) once daily for 4 days prior to sacrifice. For BrdU injections, mice were injected IP with 1.0mg of sterile-filtered Bromodeoxyuridine (BrdU) in 200ul PBS once daily for 1 to 3 days, as indicated, prior to sacrifice.

BrdU and caspase-3 intracellular staining

BrdU and activated caspase-3 staining were performed as per the manufacturers' instructions (BD Biosciences).

Inhibition of cellular division in vitro via paclitaxel

Purified mixtures of mature MZ/Fo were stimulated at 1 million/cells per ml with complete medium containing LPS (15ug/ml), IL-4 (30ng/ml), and paclitaxel (5nM) at hr 0 and aliquoted into individual wells of a 96-well plate (100ul each) to assure equal cellular number. Samples were taken in triplicate at the times indicated.

Adoptive transfers of *bCAML* and *eCAML* CD19+ cells

We sacrificed 3 mice each of *CAMLfl/fl*, *bCAML*−/−, *CAML*+/+, *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 with nothing added. CD19+ cells from each mouse genotype were injected into 5–6 male NOD/SCID mice (ages: 20–32 weeks) at 5.25-7 million cells per mouse. NOD/SCID mice were sacrificed after three weeks and the numbers of splenic CD4− CD8− B220+ CD19+ B cells assessed. Total cell number per mouse was adjusted to assume 7 million cells initially transferred.

Statistical analysis

All p values are for Student's t-tests, one tailed with assumption of homoscedastic distribution, and all error bars are for standard deviation, unless otherwise noted. Error bars labeled as standard error of the mean are for sample number (n) equal to or greater than 10 and calculated as: (standard deviation / square root of n). p values of less than 0.05 (<5% probability of null hypothesis) were considered statistically significant.

Results

bCAML−/− Mice Exhibit a 75% Loss of Mature Splenic Follicular B cells

We generated B cell-specific CAML knock-out mice (referred to as *bCAML*−/−) by crossing the CD19-cre transgene (13) into CAML fl/fl animals (8). Expression of CAML was successfully ablated, as determined by western blotting of lysates from CD19+ splenocytes from CAML-deleting (*bCAML*−/−) and littermate control ($CAML^{fl/fl}$) 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).

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

In $CAML^{fl/fl}$ mice, CD19+ splenic populations were approximately 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% ($p<1.0\times10^{-6}$). 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 ($p<3\times10^{-8}$) (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 if the loss of CAML similarly affected peritoneal B cells, we isolated and identified 3 populations (B1a, B1b, and B2) from the peritoneum based on expression of IgM, B220, CD5 (Ly-1), and CD19 (17, 18). Compared to littermate controls, *bCAML*−/− mice exhibited a reduction in the fetal liver-generated, self-renewing B220lo CD19hi B1 cells (19) and mature circulating B220hi CD19lo 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 CD5lo/neg B1b fraction, which was reduced by 77% ($p<2.5\times10^{-5}$). The CD5+ 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. Together, these data indicate that loss of CAML causes an approximately 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 *bCAML*−/− 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 and 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. On the other hand, MHCII expression was found to be reproducibly increased in the absence of CAML (Fig. 2), similarly to that reported for myb-deficient and inducible recombination-activating gene (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. Additionally, 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 raised 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.

Lymph Node B Cells Fail to Accumulate in *bCAML*−/− Mice

Reduced numbers of Fo cells might result from decreased production or accelerated loss due to impaired survival. Normally, circulating lymph node (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. On the other hand, an effect of CAML on survival would be suggested by a failure of B cells to accumulate to normal levels, since 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 5–76 weeks. Inguinal LN cells were isolated and stained with B220 and CD3 antibodies to determine the proportion of B and T cells, respectively (Fig. 3A). In both *bCAML*−/− and control mice there was an increase in B cells in the lymph nodes over time. However, at 5 weeks of age,

B cells were only 3.8% of cells within the LN of *bCAML*−/− mice compared to 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 in *bCAML*−/−mice never accumulated to proportions equivalent to controls and accounted for less than 12% of LN cells, whereas $CAML^{fl/fl}$ LN B cells were always greater than 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-hour intervals to allow for identification of newly generated cells, and were euthanized 1 to 3 days later. Cells from the spleen were fixed and stained for BrdU, and quantified by FACS. At all time points, CAML-deficient B220+ cells had higher percentages of BrdU positivity, compared to cells from control littermates ($p<0.035$), whereas B220− cells were not different between *bCAML*−/− and control mice (Fig. 3B). The twenty-four hour BrdU incorporation time point is thought to be most reflective of homeostatic proliferation within the mature splenic B cell compartment, since transitional B cells are non-dividing and bone marrow precursors cannot reach the spleen within this time interval (17). At 2 days, the transitional B cells from the bone marrow were almost entirely BrdU+, accounting for a large portion of the increase in BrdU+ B220+ cells (data not shown). Thus, we conclude that CAML-deficient B cells indeed have accelerated turnover, due to 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 day 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−/−* Mice

Histologic sections from $CAML^{fl/fl}$ and *bCAML*−/− spleens were fixed and stained with hematoxylin and eosin (H&E) to determine if the loss of Fo cells had an effect on the architecture of the organ (Supplementary Figure 1). $CAML^{fl/fl}$ spleens (left panels) had multiple well-defined darker-staining follicles (representative follicles indicated with arrows) with easily identifiable central arteries (22). By contrast, *bCAML*−/− (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*−/− mice showed a disrupted splenic architecture, due to 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 & IL4 *in vitro*

CD19-positive B cells purified from bCAML−/− and control mouse spleens were stimulated in vitro using IgM specific antibody. 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 B cell receptor activation (Fig. 4A). Next, purified B cells were stained with CFSE, and tested in vitro for response to stimulation with LPS & IL4. Cellular proliferation was determined based on CFSE dilution 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 IL4 was significantly better than that of CAML-deficient cells at all timepoints ($p<0.01$) (Fig 5A). Treatment with BAFF induced the greatest survival in both *CAMLfl/fl* and *bCAML*−/− Fo cells, and both BAFF-stimulated groups showed significant survival compared to unstimulated cells ($p<0.006$). Interestingly, *bCAML*−/− and control Fo cells displayed equal amounts of survival at 36 hours after BAFF stimulation, indicating an equivalent response. However, at later time points, *bCAML*−/− cells underwent a significant reduction compared to 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, in order 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 hours of stimulation was the same regardless of CAML genotype, similar to that seen with BAFF stimulation. However, by 70–74 hours, average *bCAML*−/− Fo cell numbers were reduced by 80% compared to $CAML^{fl/fl}$ cells ($p<0.0002$) (Fig. 5B).

To determine whether cells were dying via apoptosis, we assessed the percentage of early apoptotic (annexinV+) and dead (annexinV+ PI+) Fo cells that were present after 30 hrs in culture with LPS & IL4. CAML-deficient Fo cells exhibited a 19% decrease in overall survival compared to controls (data not shown). Furthermore, $CAML^{fl/fl}$ 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 hours of LPS stimulation, CAML-deficient B cells had higher levels of active caspase-3 ($p<0.0029$) than control cells (11 vs. 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 of drug that would suppress LPS & IL4 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 & IL4 in the presence of 5nM paclitaxel. We observed a modest initial decrease in live cell numbers of both control and *bCAML*−/− Fo B cells at 30 hrs, however there was no significant difference in survival after 30 and 50 hours in culture (Fig. 6C). After 80 hours in culture, control Fo cells maintained numbers of surviving cells similar to those at 50hr, indicating strong continued survival in the absence of proliferation. On the other hand, CAML-deficient Fo cells underwent a dramatic reduction in survival by 80 hours of stimulation ($p<0.05$). 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

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 *bCAML*−/− 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 *eCAML*−/−, were treated with tamoxifen to activate CAML gene excision. After 4 days of treatment, purified splenic MZ and Fo cells demonstrated efficient loss of CAML protein (Fig. 7A). At this early time point, *eCAML*−/− (CAML-deficient) Fo cells were only minimally reduced in number (to 73% of $CAML^{+/+}$ control mice) (Fig. 7B). Next, cells were stimulated in vitro with LPS & IL4 to evaluate effects on survival. We again found a dramatic loss of cell viability after 74 to 80 hours of stimulation, with *eCAML*−/− cells demonstrating an 80% reduction in number, compared to similarly treated control cells (Fig. 7C). We observed an intermediate effect in cells from *eCAML*+/− (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 IL4.

Next, we revisited the question of BAFF signaling by examining the ability of BAFF to up-regulate the cell surface proteins CD21, and MHC class II, which are known to be directly induced by this cytokine (25) (26). Purified B cells from tamoxifen-treated *eCAML*−/− or littermate control mice were incubated with or without BAFF in vitro for 12 hours, and then stained and analyzed by FACS. BAFF treatment induced a robust up-regulation 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.

bCAML and eCAML CD19+ Cells Show Reduced Survival After Adoptive Transfer

We next tested both *bCAML*−/− and *eCAML*−/− 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+ cells from *CAMLfl/fl*, *bCAML*−/−, *CAML*+/+, *eCAML*+/−, and *eCAML*−/− 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 CD4/8-negative B220+ CD19+) was assessed via FACS. Recovered B splenocytes from *bCAML*−/− mice showed significantly reduced (p=0.01) numbers compared to *CAMLfl/fl* (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, *eCAML*−/− mice showed an even greater reduction in number compared to *CAML*+/+ controls (p=0.00005) (Fig. 9). Heterozygote *eCAML*+/− mice displayed showed no statistically significant differences from *CAML*+/+ controls, but were likewise more numerous (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 Follicular Cells

We report here 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 lymph node Fo cells, accompanied by more rapid BrdU incorporation, indicative of faster proliferation in *bCAML*−/− mice. *In vitro*, B cells lacking CAML initially responded normally to BAFF treatment, but ultimately died in greater numbers by 90 hours of culture. Their failure to survive long term following stimulation with LPS + IL4 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 (SAC) (24). However, this effect appears to be specific to 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 hrs post stimulation, while 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 (ES) cells, which appear to have both normal proliferation and survival characteristics regardless of CAML presence or absence (10).

B Cell Receptor Signaling is Intact in the Absence of CAML

Induced deletion or defects in the B Cell Receptor (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 Ca2+ 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 hour time points, compared to unstimulated cells. It was only in long-term (> 3 day) 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*−/−, as they almost completely lack T2, MZ, and Fo cells (5). A/WySnJ mice, which bear a disrupting mutation in BAFF-R (28), exhibit an approximately 90% reduction in mature splenic B cells and a shortened B-2 B cell half-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-deficency 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 or not 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*., the major role of CAML was found to be its involvement in recycling of EGFR for epithelioid cells (10) and serving as a negative regulator of p56lck during thymocyte development (8). Likewise Liu, *et al*., found mitotic spindle defects in CAML-deleted mouse embryonic fibroblasts (MEFs) (24). Whether mature cells, many of which are non-dividing, 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 & IL4. Instead, mature cell function was dependent upon CAML expression, such that even heterozygotes displayed a significant survival disadvantage compared to 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 & IL4 in a strikingly similar manner.

Splenic Fo cells in the absence of antigen stimulation and normal bone marrow production are mostly naive, non-dividing cells. On the other hand, cells transferred into lymphopenic hosts would be subject to enhanced proliferation due to 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 in order to inhibit the apoptosis of infected cells. A novel protein from Kaposi's sarcoma-associated herpesvirus (KSHV) named K7 was identified by Feng, *et al*., 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 (TG), a pro-apoptotic 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*., discovered that the adenovirus E3-6.7 protein bore sequence homology to TACI and that it bound to CAML as revealed by yeast two-hybrid and co-immunoprecipitation experiments (12). 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 anti-apoptotic properties to TG-treated cells.

Here, 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 pro-apoptotic 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 in order 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

BAFF	B cell activating factor
BAFF-R	BAFF receptor

Ca^{2+}	calcium
CAML	calcium-modulating cyclophilin ligand
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
Fo	follicular B cell
Fr	fraction
MZ	marginal zone B cell
T1	transitional B cell type 1
T2	transitional B cell type 2
TACI	Transmembrane Activator and CAML-Interactor

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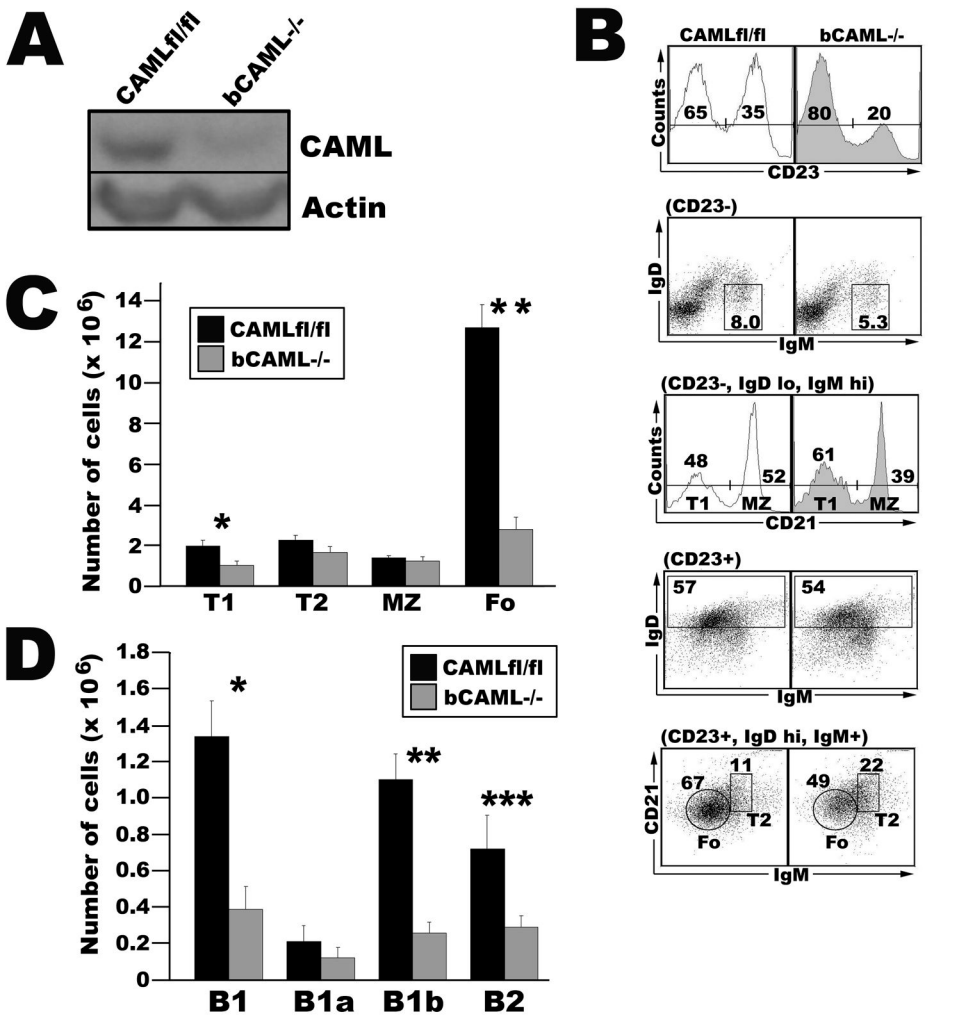

Figure 1. Abnormal Splenic B Cell Populations in *bCAML−/−* mice

(A) Cellular expression of CAML and actin proteins in CD19+ splenocytes from 13-week-old *bCAML−/−* (B cell-specific CAML deletion) and $CAML^{fl/fl}$ (littermate control) mice was assayed via western blot.

(B) Flow cytometric analysis of splenic B cell populations in *bCAML−/−* and $CAML^{fl/fl}$ littermate controls. Cells stained for CD21, CD23, IgD, and IgM. Values indicate percent of whole spleen or gated populations. T1 = Transitional 1, T2 = Transitional 2, MZ = Marginal Zone, Fo = Follicular. Plots are representative of all splenocyte analyses.

(C) Average numbers of splenic B cell populations from 13 *bCAML−/−* and 12 $CAML^{fl/fl}$ littermates, 10 to 22 weeks old, from at least 3 separate experiments. Error bars represent standard error of the mean. *$p<0.005$, **$p<3\times10^{-8}$.

(D) Peritoneal B cell populations from 10 *bCAML−/−* and 11 $CAML^{fl/fl}$ littermates, 17–23 weeks old. B1 population shown in total and divided into B1a and B1b populations as labeled. Error bars represent standard error of the mean. * $p<0.0004$, ** $p<2.5\times10^{-5}$, *** $p<0.025$.

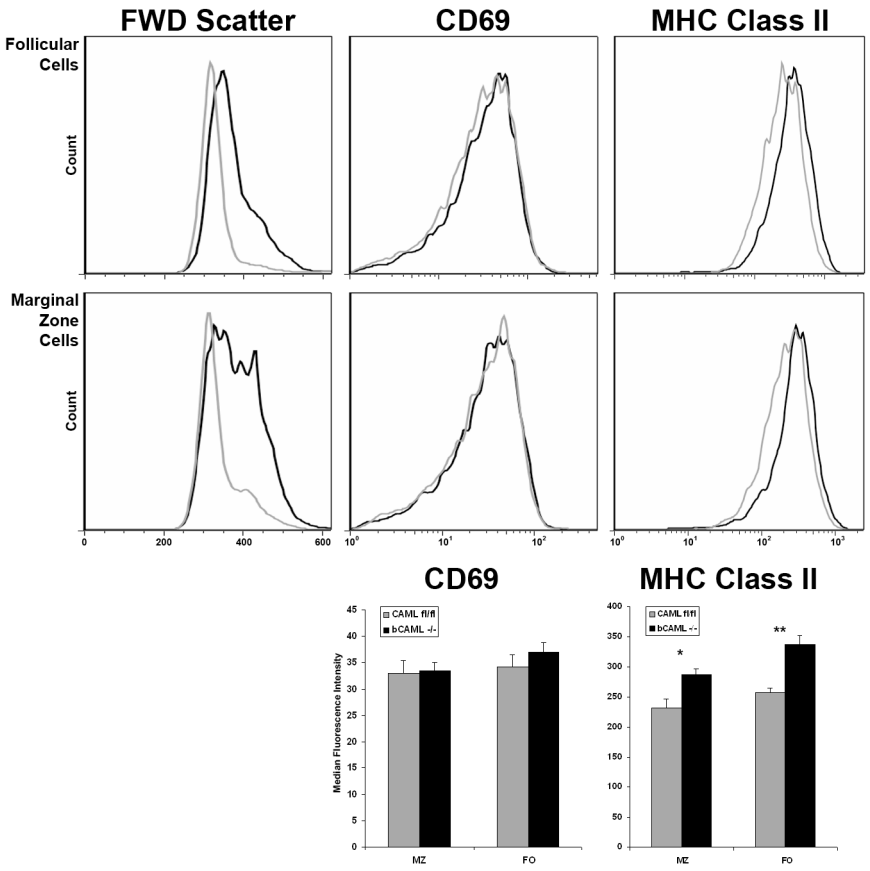

Figure 2. *bCAML*−/− Splenic B cells Display Increased size and MHC II expression

Representative histograms showing forward scatter and expression of cell surface CD69 and MHC class II by *bCAML*−/− (black) and CAML$^{fl/fl}$ (gray) splenic F0 and MZ cells as indicated. Graphs show the average Mean Fluorescence Intensities of CD69 and MHC class II expression by CAML fl/fl (gray) and bCAML−/− (black) MZ and Fo cells as indicated. * $p<0.022$, ** $p<0.012$

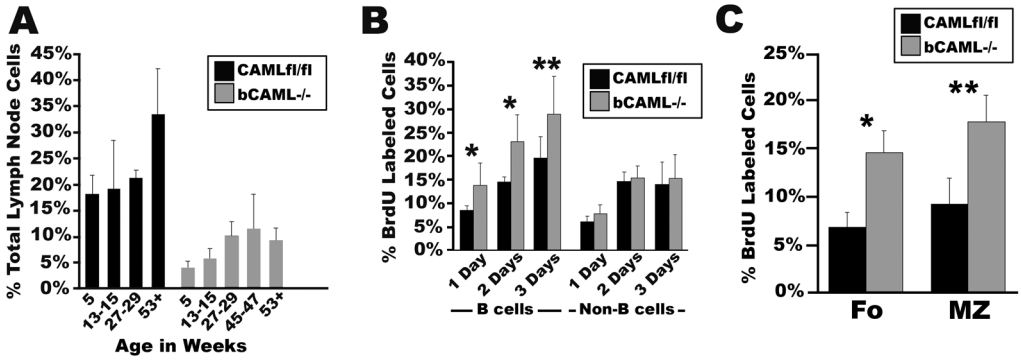

Figure 3. *bCAML−/−* Cells Fail to Accumulate in the Periphery and Display Higher Turnover Rates in the Spleen

(A) Percent of B220+ cells in inguinal lymph nodes from $CAML^{fl/fl}$ (black bars) and *bCAML−/−* (grey bars). Ages of mice in weeks listed on X axis. Each bar represents a minimum of 3 mice.

(B) Average percent of splenic BrdU+ cells after 1–3 days in $CAML^{fl/fl}$ (black bars) & *bCAML−/−* (grey bars) mice, aged 10–15 weeks. *p<0.035, **p<0.025 Each bar represents a minimum of 3 mice.

(C) Average percent of BrdU+ cells in mature splenic B cell populations (Fo=Follicular MZ=Marginal Zone) after 2 days in $CAML^{fl/fl}$ (black bars) & *bCAML−/−* (grey bars) mice, aged 15 weeks. *p<0.0035, ** p<0.0091. (n=3 each)

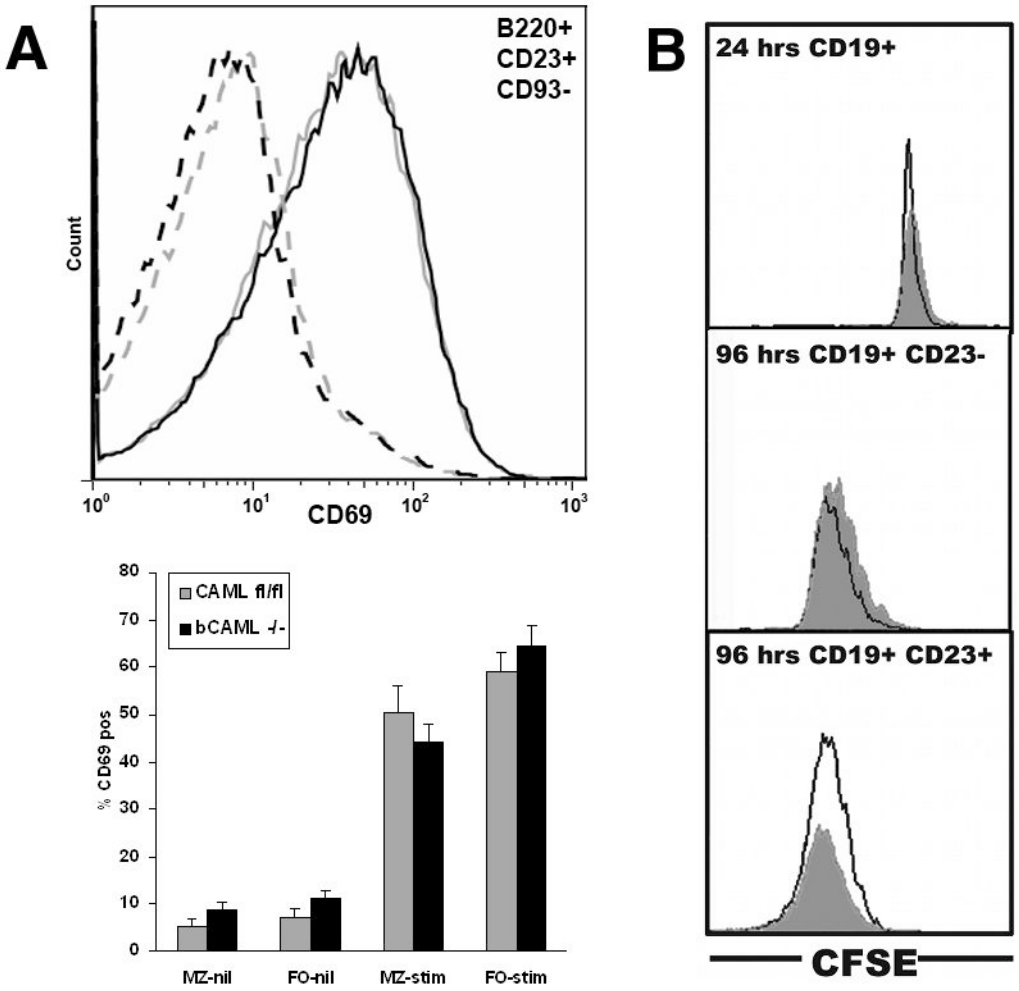

Figure 4. Normal activation and proliferation in *bCAML−/−* Cells

(A) Positively selected CD19+ splenocytes were stimulated with IgM antibodies or nil for 24 hours, and then analyzed by FACS for induction of surface CD69. (Top panel: representative FACS plots for Fo cells) (B) Positively selected CD19+ splenocytes were stained with CFSE and stimulated with LPS & IL4. $CAML^{fl/fl}$ (black line, unfilled) and *bCAML−/−* (grey filled). Histograms represent CD19+ population after 24hrs (top panel) or CD19+ CD23− (MZ) and CD19+ CD23+ (Fo) 96hrs after stimulation. (data representative of 3 mice each)

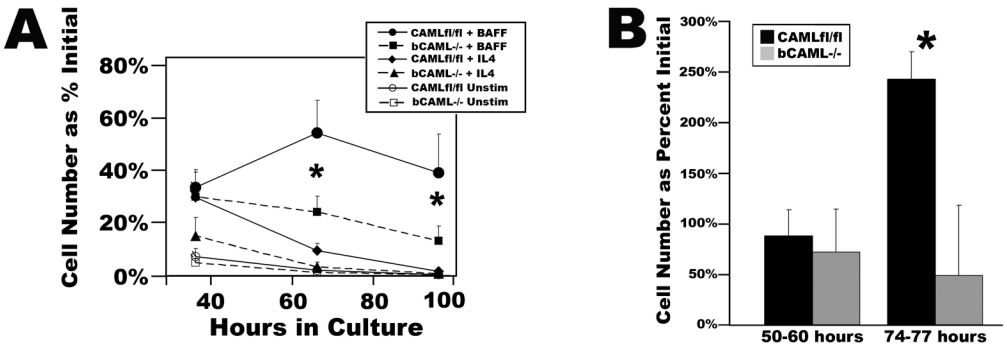

Figure 5. *bCAML−/−* Cells Exhibit Long-Term Survival Defects

(A) Fo cells from 24-week-old CAML$^{fl/fl}$ (n=3) and *bCAML−/−* (n=5) mice were cultured for 96 hours without stimulation or stimulated with 50ng/ml IL4 or 100ug/ml BAFF. Unstimulated: CAML$^{fl/fl}$ (solid line, open circles) and *bCAML−/−* (dashed line, open squares), IL4 stimulated: CAML$^{fl/fl}$ (solid line, closed diamonds) and *bCAML−/−* (dashed line, closed triangles). BAFF stimulated: CAML$^{fl/fl}$ (solid line, closed circles) and *bCAML−/−* (dashed line, closed squares); *=p<0.006. Number of live cells at each timepoint was divided by initial number placed in culture.

(B) *In vitro* number of splenic Follicular cells from 4 CAML$^{fl/fl}$ (black bar) and 8 *bCAML−/−* (grey bar) mice, aged 15–22 weeks, stimulated with LPS and IL4 after 50–60 hours and 74–80 hours in culture. Fo cell number is graphed as the number of cells at each time divided by the initial number cultured. Data representative of 2 separate experiments. *p<0.0002.

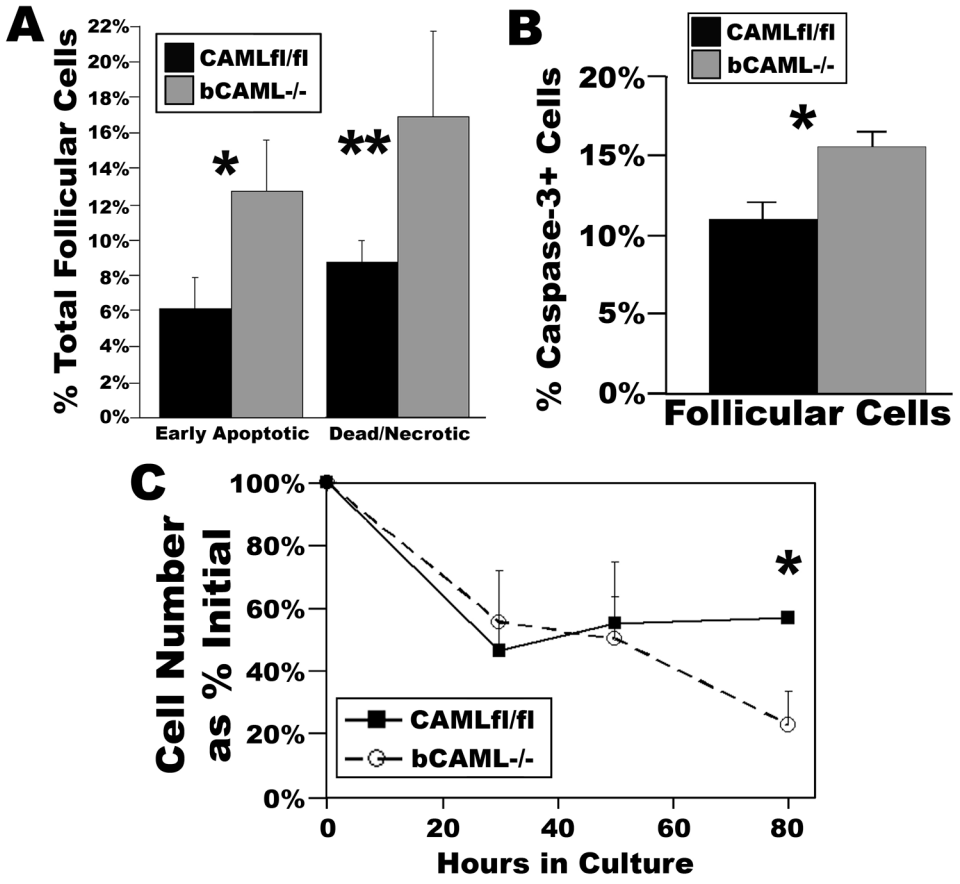

Figure 6. *bCAML−/−* Cells Die via Apoptosis

(A) Percentage of early apoptotic (AnnexinV+) and dead (AnnexinV+ and PI+) CD23+ Fo cells from 15 to 21 week-old mice, after 30 hrs in culture after LPS& IL4 stimulation. *p<0.0037, **p<0.0081 (n=4 each)

(B) Intracellular caspase-3 staining in mature splenic B cells from 17-week-old CAML$^{fl/fl}$ and *bCAML−/−* mice, stimulated with LPS only and cultured for 55 hours. *p<0.0029 (n=3 each)

(C) Survival of CAML$^{fl/fl}$ and *bCAML−/−* Fo cells from 11 to 13-week-old mice, cultured with paclitaxel, LPS, & IL4. Graphed as the number of cells alive at each timepoint divided by the number initially cultured. CAML$^{fl/fl}$ = solid lines & black squares; *bCAML−/−* = dashed lines & open circles. *p<0.0004 (n=4 each)

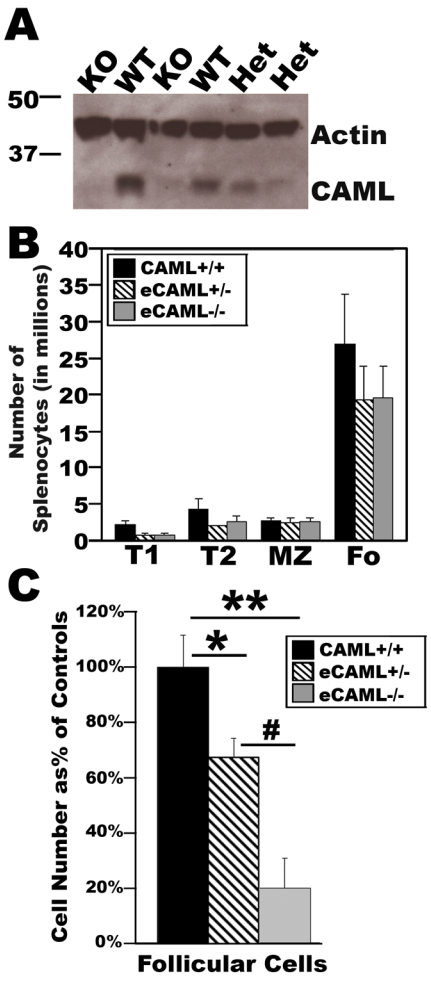

Figure 7. Defective Survival in Mature Fo cells due to Induced Loss of CAML

(A) Cellular expression of CAML and actin proteins from mature MZ and Fo B cells was assayed via western blot. Mouse genotypes are labeled as follows: CAML+/+ = WT, *eCAML*+/− = Heterozygote (Het), *eCAML*−/− = knock-out (KO) Mice were 13 weeks old.

(B) Flow cytometric analysis of splenic B cell populations in CAML+/+ (black bars) *eCAML*+/− (striped bars) and *eCAML*−/− (grey bars) littermates, 2 mice each, aged 13 weeks. T1 = Transitional 1, T2 = Transitional 2, MZ = Marginal Zone, Fo = Follicular. Data representative of 2 separate experiments.

(C) Numbers of splenic Fo cells after 74–80 hours in culture with LPS & IL4. Cell number is graphed with average control Fo cell survival from each experiment equal to 100% and all individual data points divided by control Fo cell number. CAML+/+ (black bars, n=6) *eCAML*+/− (striped bars, n=4) and *eCAML*−/− (grey bars, n=4). Mice aged 13–27 weeks. *p< $p<2.01\times10^{-6}$, **p<0.00052, #p<0.00014.

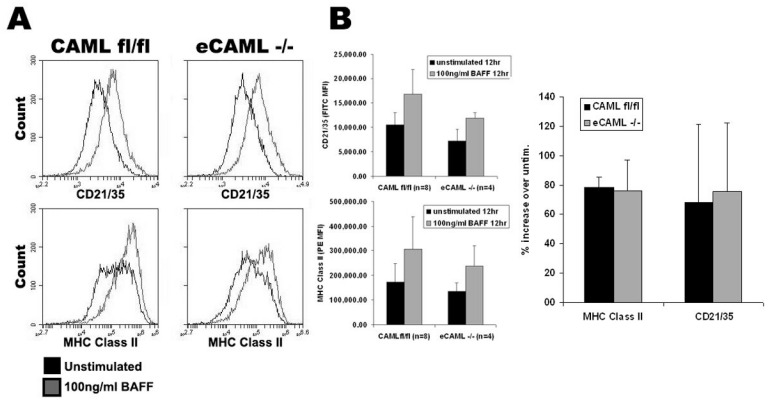

Figure 8. BAFF increases expression of CD21/35 and MHCII despite loss of CAML

Purified B cells from tamoxifen-treated eCAML−/− or littermate control mice were incubated in vitro for 12 hours with or without recombinant BAFF (100 ng/ml). Fo cells were analyzed for expression of CD21/35 and MHC class II 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).

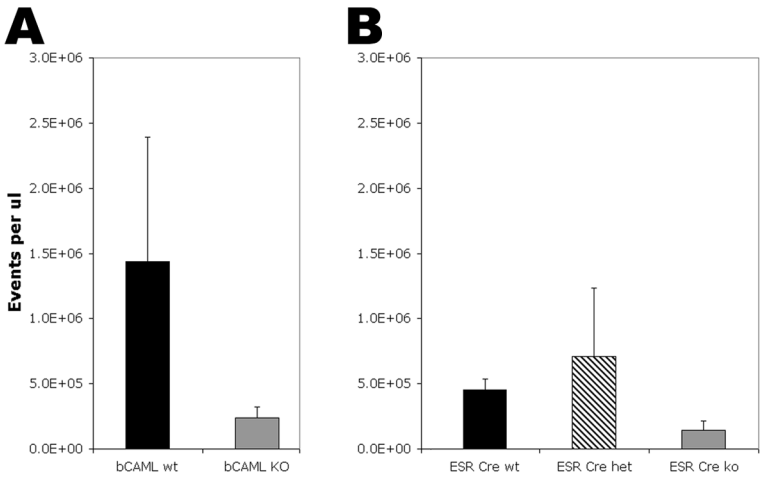

Figure 9. Reduced Survival of CAML-Deficient B Splenocytes Following Adoptive Transfer

(A) Total number of B splenocytes recovered NOD-SCID mice after adoptive transfer of CD19+ splenocytes from CAMLfl/fl (black bars n=6) or bCAML−/− (grey bars, n=5). Cell number recovered is adjusted to account for the 7 million cells initially transferred.

(B) Total number of B splenocytes recovered NOD-SCID mice after adoptive transfer of CD19+ splenocytes from CAML+/+ (black bars n=6), eCAML+/− (striped bars, n=6), and eCAML−/− (grey bars, n=5).