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A Posttranscriptional Pathway of CD40 Ligand mRNA Stability Is Required for the Development of an Optimal Humoral Immune Response

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CD40 ligand (CD40L) mRNA stability is dependent on an activation-induced pathway that is mediated by the binding complexes containing the multifunctional RNA-binding protein, poly(pyrimidinyl)tract-binding protein 1 (PTBP1) to a 3′ untranslated region of the transcript. To understand the relationship between regulated CD40L and the requirement for variated expression during a T-dependent response, we engineered a mouse lacking the CD40L stability element (CD40LΔ5) and asked how this mutation altered multiple aspects of the humoral immunity. We found that CD40LΔ5 mice expressed CD40L at 60% wildtype levels, and lowered expression corresponded to significantly decreased levels of T-dependent Abs, loss of germinal center (GC) B cells and a disorganized GC structure. Gene expression analysis of B cells from CD40LΔ5 mice revealed that genes associated with cell cycle and DNA replication were significantly downregulated and genes linked to apoptosis upregulated. Importantly, somatic hypermutation was relatively unaffected although the number of cells expressing high-affinity Abs was greatly reduced. Additionally, a significant loss of plasmablasts and early memory B cell precursors as a percentage of total GL7+ B cells was observed, indicating that differentiation cues leading to the development of post-GC subsets was highly dependent on a threshold level of CD40L. Thus, regulated mRNA stability plays an integral role in the optimization of humoral immunity by allowing for a dynamic level of CD40L expression on CD4 T cells that results in the proliferation and differentiation of pre-GC and GC B cells into functional subsets. The Journal of Immunology, 2021, 206: 2552–2565.

At early time points in an immune response CD40/CD40 ligand (CD40L) engagement leads to the rapid proliferation and differentiation of Ag-selected B cells into short-lived extrafollicular plasmablasts or founder cells that seed germinal centers (GCs) (1–4). Importantly, enhanced CD40 signaling at this early stage of the response results in a preferential shift toward plasmablast differentiation and limits B cell entrance into the follicles (5–8). CXCR5+ T cells that preferentially help B cells produce Abs can be visualized using intravital two-photon imaging as early T follicular helper (TFH) cell–B cell interactions at the border of the B cell follicles (9–11). The TFH cells require BCL-6 and are physically located in the follicle where they are critical for establishing GCs, selecting high-affinity B cells, and modulating the differentiation of cells into plasma and memory subsets (12). These GC processes are highly dependent on the dynamic signaling between CD40L and CD40 that may flux in amplitude and duration throughout the course of the response (13). Delivering sufficient levels of CD40L to Ag-selected B cells is carried out by long-lived immunologcal synapses of T-B interactions at the T zone–follicle border allowing the B cells to expand and form GCs. In contrast, T-B interactions inside GCs are of relatively short duration with a few

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Abbreviations used in this article: ASC, Ab-secreting cell; BM, bone marrow; CD40L, CD40 ligand; CSR, class switch recombination; DZ, dark zone; DFC, follicular dendritic cell; Frag., fragment; GC, germinal center; GO, Gene Ontology; LLPC, long-lived plasma cell; LZ, light zone; NP-KLH, 4-hydroxy-3-nitropheryl-acetyl-keyhole limpet hemocyanin; pBS, phBlueScript; PTBP1, poly(pyrimidinyl)tract-binding protein 1; qRT-PCR, quantitative RT-PCR; RT, room temperature; SHM, somatic hypermutation; SRBC, sheep RBC; TD, T-dependent; TFH, T follicular helper; UTR, untranslated region; WT, wildtype.

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mutations in CD40L (5, 14, 16, 18, 19, 22–24). The importance of context-specific bidirectional signaling between GC B cells and T cells to collectively shape the quality of a GC response is evident. However, our understanding of pathways that regulate the expression of CD40L throughout the course of a GC response is limited.

It has previously shown that CD40L mRNA expression in CD4 T cells is dynamically regulated by an activation-induced process of mRNA stability mediated by complexes containing the polyuridine tract-binding protein 1 (PTBP1) (25–28). Whereas PTBP1 is most well characterized as playing a major role in splice-site selection of a diverse set of transcripts in many different cell types, it is also known to be critical for multiple steps of mRNA biosynthesis including polyadenylation, transport, stability, and initiation of protein translation (29, 30). PTBP1 consists of four RNA recognition motifs connected by unstructured linker regions that bind short CU sequences contained within a longer pyrimidine tract (31, 32). In addition to PTBP1, two PTB paralogs are expressed in mammalian tissues: PTBP2, which is expressed principally in neurons (33) and ROD1 or PTBP3, expressed preferentially in hematopoietic cells (34). Recent work targeting PTBP1 and PTBP2 in B cells revealed that PTBP1 plays an essential role in the development or maintenance of mature B cells, and if deleted is compensated by the upregulation of PTBP2 (35).

Our previous studies that analyzed the expression of CD40L in vivo CD4 T cells revealed that the mRNA was unstable at early times of activation followed by an increase in transcript stability at extended times of activation (25, 27). We further showed that the stability phase of this process was mediated by two PTBP1-containing complexes (termed Complex I and II) that bound to three distinct sites (A–C) within the CD40L 3′ untranslated region (UTR) (26, 27, 36). Although Complex I, which also contains nucleolin, is critical for protecting the mRNA from decay, Complex II is likely involved in translational control of CD40L through hnRNPL, another component of Complex II (37, 38). The possibility that other proteins, including the PTB paralogs, are important for stability complex formation or that under certain circumstances can substitute for PTBP1 has not been resolved. The binding of Complex I to CD40L mRNA was found to correspond to a phosphorylation pattern unique to the cytoplasmic fraction of PTBP1 at late times of activation, although exact amino acids or the kinase(s) that target these sites have yet to be determined (28). Finally, PTBP1 knockdown in human T cells resulted in decreased CD40L expression, diminished proliferation as well as reduced activity through PLCγ1/ERK1/2 and the NF-kB signaling pathways (39).

From these earlier studies it was clear that regulated mRNA decay was critical for modulating CD40L expression, however, the significance of this regulatory pathway within the physiological context of a developing immune response was unknown. Thus, in this work, we report the development of a mouse model of reduced CD40L expression that is based on mutating the mRNA stability element (termed CD40LΔ5). This model was used to determine how the stability pathway of CD40L expression functioned in critical biological events within an emerging T-dependent (TD) immune response. Our findings clearly demonstrate that an intact CD40L stability pathway is essential for optimal production of GC-specific B cells and the generation of IgM and IgG plasmablasts and precursor B memory cells. Additionally, significant loss of GC-specific B cells corresponded to a disrupted GC architecture and an altered distribution of cells between the LZ and DZ. Whereas many of the observed changes could be explained by a decrease in the number of GL7+ B cells due to reduced proliferation and increased apoptosis, the overwhelming reduction of differentiated precursor memory and plasmablast pools suggested an additional and important requirement for precise levels of CD40L expression in determining GC output. Thus, regulated mRNA stability plays a major role in maintaining the amplitude of CD40L expression on CD4 T cells that is essential for driving optimal B cell proliferation and differentiation in a humoral immune response.

Materials and Methods

Generation of the CD40LΔ5-targeting construct

CD40L Frag. 2 (1418-bp fragment [Frag.] corresponding to the 3′ region of exon 5 [Gene Bank Accession no. AL672128.8, bp 149859–151162] plus downstream sequences [bp 151163–152777]) were PCR amplified from C57BL/6 genomic DNA using Phusion Polymerase (Promega) and 0.2μM Frag. 2 primers (all primers listed in Supplemental Table II). This Frag. was subcloned into the pGem-T Easy vector using the T-A cloning and site-directed mutagenesis was carried out targeting 178 bp within the CD40L stability element corresponding to repetitive CU and CA stretches in binding sites I and II, respectively (150812–150990). CD40L Frag. 1 (bp 145352–149859) and 3 (bp 151278–155100) were PCR amplified and cloned into pBluescript (pBS) using Not I and Pst I sites (Frag. 1) and Hind III and Not I sites (Frag. 3) to generate pBS-Frag-1 and pBS-Frag-3, pBS-Frag-1 and Frag. 2 were combined into pBS-Frag.1 + 2, and the final targeting construct was generated by combining pBS-Frag.1 + 2 and pBS-Frag.3 into the pEASY Flox vector [Addgene plasmid no. 11725; http://n2t.net/addgene:11725; RRID: Addgene 11725 (40)] to generate pEASY-Complete. The genetic organization was confirmed by restriction enzyme analysis and PCR. The fidelity of all exons was confirmed by sequencing.

Generation of CD40LΔ5 mice

Generation of knock-in mice harboring the pEasy-Complete vector was carried out by the Rodent Genetic Engineering Laboratory at New York University Langone (New York, NY). Gene targeting was performed using proprietary C57BL/6 Embryonic Stem Cell lines. A total of 200 positive clones were screened for the presence of the targeted allele. Seven positive clones were analyzed by PCR and Southern blot analysis, and positive clones were introduced by microinjection into C57BL/6 tetraploid blastocysts, which resulted in the generation of fully ESC-derived mice. The neomycin-resistance gene (NeoR) was removed by crossing mice carrying the targeted allele to a Cre-deleter transgenic male and resultant mice carrying the Cre gene were backcrossed several generations until the Cre gene was no longer detectable by PCR. Only hemizygous males (Y/+, Y/Δ5) and homozygous females (Y+/Y, Δ5/Δ5) were used in these studies, and both males and females were included in all experiments, where possible. Because of the location of the mutation on the X chromosome, wildtype (WT) female mice and Δ5/Δ5 females were not able to be generated from the same litter, but were generated from breeding male Y/+ or Y/Δ5 to female heterozygotes. All animals were caged within the same room. Mice were housed in ventilated microisolators under specific pathogen–free conditions in a Rutgers University mouse facility and used at 6–10 wk of age in accordance with National Institutes of Health guidelines and under an animal protocol approved by the Animal Care and Use Committee of Rutgers University.

Immunization

Sheep RBCs (SRBC) (Innovative Research, Novi, MI) were resuspended at a ratio of 1:1 in PBS and 500 μl injected i.p. Mouse spleens were harvested at day 8 and splenic mononuclear cells dissociated, filtered, and suspended in HBSS with 5% FBS. Following RBC lysis for 35 s using 1× ammonium-chloride-potassium (ACK) lysis buffer, splenic cells were washed three times with HBSS with 5% FBS, resuspended in RPMI with 10% FBS and counted. Bone marrow (BM) cells were isolated by flushing the femurs with PBS.

4-Hydroxy-3-nitrophenyl-acetyl–keyhole limpet hemocyanin (NP-KLH; BioSource) was dissolved in sterile PBS at a concentration of 1 mg/ml. For primary immunizations and secondary boosters (at 21 d), NP-KLH was mixed with inject alum adjuvant (Thermo Scientific) at a 1:1 ratio and 200 μl alum was injected i.p. into mice. Prior to injection, blood serum was collected at day −4, and then following injection at days 7, 14, 21, and 28. Draining mesenteric lymph node cells and spleenocytes were collected from NP-immunized mice at day 28 using protocols described above.
Abs and primers

All Abs and primers used in this study are listed in Supplemental Tables I and II.

B cell morphological examination

Splenocytes from SRBC-immunized mice (day 8) were embedded and snap frozen in Optimal Cutting Temperature compound (Sakura Fintech) and stored at −80°C. Microsections (20 μm) were prepared using a CM1900 cryostat microtome (Leica) and stained with Mayer’s Hematoxylin (Sigma-Aldrich) followed by Eosin Y stain (Sigma-Aldrich). For expression of GC-specific proteins, slides were rehydrated with 1× PBS and blocked with 1× PBS + 0.1% Tween 20 (1× PBS-T) containing 1% Fc block (BD Biosciences) and 3% BSA. Sections were stained with either A594-CD4, allophycocyanin- IgGs, and A480-GITL mAbs or FITC-CD21/35, PE-CD23, and A480-CXCR4 mAbs diluted in PBS-T with 1% Fc block. After incubation at room temperature (RT) for 1 h, slides were washed with PBS-T and mounted, and bright-field micrographs of sections were imaged using a Nikon Eclipse E600.

Flow cytometry

Single-cell suspensions (1×10^6 cells) from spleen, lymph nodes, thymus, and BM were stained with Zombie-NIR fixable viability dye (BioLegend) for 15 min, washed, and suspended in PBS with 1% BSA, 2% rat serum, and 1 μg/ml of anti-FcγR to block FcR binding. After 10 min of incubation on ice, the appropriate primary Abs, unconjugated or conjugated to different fluorophore markers, were added to the cells at a concentration 1–10 μg/ml and incubated for 30 min on ice. The cells were washed two times with PBS + 1% BSA, and secondary Abs were added if needed for 30 min on ice. The cells were washed two times in PBS + 1% BSA and fixed with 1% paraformaldehyde. For staining of intracellular Ags, fixed cells were either incubated with 0.1% Triton-X for 5 min and washed twice before staining or maintained in Cytofix/Perm buffer (BD Biosciences) for the duration of staining. Flow cytometric analysis was performed on an FACS Calibur or Cytek flow cytometric data.

Isolation of cells

CD19^+ B cells were isolated from splenocytes using the Mojosort Positive Selection Kit (BioLegend). Briefly, splenocytes were harvested from NP-KLH challenged and boosted mice at 28 d and treated with 10 μg/ml cycloheximide for 1 h prior to and during activation with 50 ng/ml PMA and 1 μg/ml ionomycin at 37°C for 30 min. CD14^-T cells were identified using CD4-FITC Ab and cells expressing CD10 with CD40L-PE.

ELISpot detection of NP-specific Ab forming cells (AFC)

Multiseed 96-well assay plates (MilliporeSigma) were pretreated with 15 μl of 35% EtOH per well for 1 min. The plates were washed and coated with 100 μl of NP_20-25 BSA (50 μg/ml in PBS) and incubated at 4°C overnight. Plates were blocked with 100 μl per well RPMI + 10% FBS for 2 h at 37°C. Two-fold dilutions of cells starting from 10^6 cells per 100 μl were added to the ELISpot plate and samples incubated overnight at 37°C and 5% CO2. The plates were washed six times with 1× PBS-T. A total of 100 μl of AP-conjugated anti-mouse IgG1 (1 μg/ml) was added to the plate at a dilution of 1:2000 in PBS with 2% BSA and incubated at RT for 1 h. Following incubation, the plates were washed three times with 1× PBS-T, and an additional three times with PBS. Ready to use NBT/BCIP substrate buffer was added at 100 μl per well and incubated 5–10 min. Spot development was stopped by washing under running water and counted after 24 h.

Measurement of Ab responses

Serial dilutions of anti-NP Abs in immune sera were assayed by ELISA using either NP_20-25 BSA (high valency) or NP1 (low valency to restrict binding to the high-affinity Ab). Briefly, 96-well plates (Nunc Maxisorb) were coated with 50 μl per well of 5 μg/ml of NP-BSA diluted in PBS. Following overnight incubation at 4°C, plates were washed three times with 1× PBS-T, blocked with PBS + 2% BSA, and incubated at RT for 1 h. Diluted samples were added to plates, incubated for 2 h at 37°C and washed three times with 1× PBS-T. Following incubation, 50 μl of detection Ab diluted 1:2000 in PBS–1% BSA was added to each well, incubated at RT for 1 h and plates washed four times with 1× PBS-T, and 50 μl of PNPF substrate solution added for 10–30 min. Plates were read on a spectrophotometer at 405 nm. The sample concentrations were interpolated from the standard curves using GraphPad Prism.

Statistical analyses were performed on GraphPad Prism software version 7.
Results

Generation of a mouse model of decreased CD40L expression

The mouse CD40L stability element was previously defined as a 350-bp region that showed high homology to a stability region in the human CD40L gene. Using the entire region as a probe in R-EMSA we found that all complexes bound to this region were competed for by anti-PTBP1 Abs suggesting that PTBP1 was the major component of RNA complexes binding to this region (42). To identify sequences within the 3' UTR that resulted in destabilization of the transcript, the different regions were cloned into the pGL3 vector and luciferase measured following transfection into Jurkat cells. We found that site B, and to a lesser extent a region equivalent to human site C, enhanced expression of luciferase, whereas site A alone or in combination with other elements had a negative effect on expression (Supplemental Fig. 1a, 1b). We therefore generated a targeting construct containing a deleted region of 178 bp, which included the regions defined as sites B and C. Following targeted recombination, thelox1 floxed Neo5 gene was removed by breeding with a Cre-deleter strain and a pure line of mutant mice lacking the stability element was generated (termed CD40LΔ5) (Supplemental Fig. 1c–f).

To understand the role of mRNA stability in providing threshold levels of CD40L for TD Ab responses and cellular fate decisions, CD40LΔ5 mice were analyzed for production and distribution of T cells and B cells prior to immunization. Distribution of thymic and splenic T cell subsets revealed no obvious differences with WT controls (Supplemental Fig. 2a). Additionally, prior to and following Ag challenge, the absolute number of B220+ B220+IgM+IgD−/−, B220+IgM−IgD+, follicular (B220+CD21hiCD23lo) and marginal zone (B220+CD21hiCD23hi) B cells were similar between CD40LΔ5 mutants and WT controls (Supplemental Fig. 2b). Thus, mice carrying the CD40LΔ5 mutation were indistinguishable from WT mice with respect to the distribution and number of lymphoid cells prior to Ag challenge.

The CD40LΔ5 mice express lower levels of CD40L on CD4 T cells

We next asked whether the expression of CD40L was decreased in CD4 T cells from mice immunized in vivo with NP-KLH followed by ex vivo stimulation with APCs presenting KLH (APC-KLH). Specifically, CD40L surface expression was found to be frequency lower in CD40LΔ5 CD4 T cells that were solely stimulated ex vivo with APCs alone although this difference was not consistently seen in all animals (Fig. 1A, 1B, left panels). Notably, CD40LΔ5 CD4 T cells that were re-stimulated ex vivo with APC-KLH expressed significantly less CD40L than WT cells and this difference was highest at 12 h after activation (Fig. 1A, 1B, right panels, and Fig. 1C). We also examined the expression of preformed CD40L in ex vivo splenic CD4 T cells (28 d after immunization and boost) following acute reactivation with PMA/ionomycin. Similar to what we observed with APC-stimulation at early time points ex vivo, there was no significant difference in this intracellular pool of preformed CD40L upon reactivation between CD40LΔ5 and WT CD4 cells (Fig. 1D).

To determine if the observed decrease in CD40L expression in CD4 T cells from CD40LΔ5 mice was consistent with altered mRNA stability, splenic CD4 T cells were isolated from NP-KLH immunized mice, stimulated ex vivo for 12 h with APC-KLH and treated with the transcriptional inhibitor DRB. Decay was measured over a time course of 90 min and cells collected and analyzed every 30 min for CD40L RNA by qRT-PCR. Compared to our findings with WT CD4 T cells, the stability of the CD40L transcript in mutant cells was reduced to a T1/2 < 30 min from ~45 min (Fig. 1E).

Finally, to confirm that the stimulation of CD4 T cells expressing the CD40LΔ5 mutation did not result in an overall decrease in activation, splenic CD4 T cells from NP-KLH immunized mice were isolated and the expression of two additional activation markers was assessed by flow cytometry and qRT-PCR. In contrast to CD40L, no significant difference in CD69 and CD25 expression was observed in CD4 T cells from CD40LΔ5 mice. This strongly suggested that loss of the stability program did not result in a global decrease in CD4 T cell activation but was confined to affecting only the expression of CD40L (Fig. 1F, 1G).

The pathway of CD40L stability is critical for the establishment of the GL7+ B cell population

Although the requirement of CD40-CD40L interactions in the formation of GCs is well documented in both mice and humans we wished to discern whether the threshold of CD40L expression provided by mRNA stability is critical for the establishment and function of these structures (43). Thus, splenic sections from mice...
injected with SRBCs were visualized using immunohistochemistry to delineate distinct structural regions of the GCs including naive B cells (IgD⁺), T cells (CD4⁺), and Ag-selected GC B cells (GL7⁺) (20, 44). Surprisingly, we found that the GC architecture in spleens from CD40LΔ5 mice was highly disorganized relative to WT mice; the IgD⁺ and CD4⁺ sectors appeared relatively normal, whereas the GL7⁺ area was smaller and positively stained cells were diffused throughout the GC (Fig. 2A, 2B). When sections were analyzed in more detail, there appeared to be little difference in the overall number of initiated GCs; however, the size of the individual GC was significantly decreased in the mutant (median: 5.9 versus 3.8, respectively) and disorganized (Fig. 2C, 2D). Further confirmation of these findings was carried out using flow cytometry to quantify the GC B cell population (CD19⁺Fas⁺GL7⁺). Again, we found the GL7⁺ B cell population was significantly reduced (2- to 3-fold) in mice expressing the CD40LΔ5 mutation (Fig. 2E).

Importantly, the frequency of CD4⁺CXCR5⁺PD-1⁻ (TFH) cells was highly similar between mutant and WT mice thus eliminating the possibility that the decreased frequency of GC B cells was linked to an overall loss of TFH cells (Fig. 2F). Although there was no significant difference in the absolute number of TFH cells, the expression of CD40L at day 8 following SRBC injection on this population was decreased by ~40% (Fig. 2G). Together, these findings are consistent with the significant drop in GC B cells from immunized CD40LΔ5 mice being a result of the lowered threshold of CD40L expression on TFH cells.

GL7⁺ GC B cells from CD40LΔ5 mice are located predominantly within the LZ

We further addressed if the disruption of the GC structure was solely caused by a decrease in the frequency of cells or if there were associated changes in the location and distribution of B cell populations within the GC. To this end we analyzed the

FIGURE 2. Disruption of the CD40L stability pathway results in disorganized GCs and a loss of GL7⁺ B cells. (A) GC structures were visualized in mice 8 d following SRBC immunization using 20 μm sections of frozen spleens stained with H&E and visualized using a Nikon Eclipse E600 Fluorescence Microscope. (B) Splenic sections were stained with anti-mouse IgD to identify naive B cells (allophycocyanin-labeled, purple), anti-mouse CD4 for T cells (PE-labeled, red), and anti-mouse GL7 for GC B cells (Alexa 488, green). (C and D) Spleens were isolated from SRBC-immunized mice (as above) and GCs identified using Olympus Studio 2 software. The number of GCs per image frame was calculated and the area of each GC quantified and compared between WT and CD40LΔ5 mice. (E) Representative flow analysis (middle) of compiled percentages and total cell number (right graphs) of CD19-gated splenocytes stained with Abs against GL7 and Fas protein, 8 d after SRBC immunization. Values are averages of WT and CD40LΔ5 mice (n = 7) where bars denote the geometric mean and SEM. (F) Representative flow cytometry data (left) and compiled percentages and total cell number (right graphs) of TFH cells (CD4⁺CXCR4⁺PD-1⁻) isolated from WT and CD40LΔ5 spleens 8 d after SRBC immunization using CD4-BV510, CXCR5-BV421, and PD-1-FITC Abs. Bars represent the geometric means of WT and CD40LΔ5 mice (n = 5) with the error bars determined by the SEM. (G) Representative histogram of CD40L expression on TFH cells and quantification using medium fluorescence intensity (MFI) at 8 d after SRBC immunization (n = 5). Bars represent the geometric mean and error bars the SEM. Significance was calculated in all graphs by unpaired, two-tailed t test in which *p ≤ 0.05, ***p ≤ 0.001. NS, not significant.
The posttranscriptional pathway of CD40LΔ5 is critical for B cell proliferation

To gain a mechanistic understanding of the relationship between altered CD40L expression and the loss and redistribution of GL7+ B cells within the GC, we carried out RNA sequencing with bead-sorted CD19+ B cells at day 8 following SRBC injection. We chose to look at this broad B cell population based on the assumption that this approach had the highest likelihood of capturing critical CD40LΔ5-dependent transcriptional changes. In contrast, using GL7+ cells exclusively as the starting material had the potential of masking early differentiation events that were dependent on CD40L levels prior to GL7 expression. Accordingly, a pattern of 95 downregulated and 99 upregulated genes were identified as being associated with CD19+ B cells from mice expressing the CD40LΔ5 mutation. Many of the genes that were downregulated mapped to pathways involved in cell cycle, DNA replication and mismatch repair. We also independently identified a number of genes associated with apoptosis that were upregulated in the B cells expressing the CD40LΔ5 mutation (Fig. 4A, 4B). Because Ag-selected GC B cells represent a small percentage of the total splenic B cells, we wished to establish whether gene expression changes associated with proliferation also extended to the GL7+ B cells. To this end splenic cells were gated on CD19 and GL7 and analyzed for expression of proliferation markers Ki-67 and phosphohistone H3 (PHH3) (47). Importantly, we observed highly reduced levels of both markers indicating that proliferation was significantly inhibited both in CD19 and GL7+ B cells from CD40LΔ5 mice (Fig. 4C, 4D). To determine whether apoptosis was also increased in the GC B cell population we measured the expression of caspase 3 and poly (ADP-ribose) polymerase or c-Parp, two proteins upregulated in apoptotic cells. CD40LΔ5 mice showed significant increases in both proteins suggesting a
FIGURE 4. Gene Expression Profiling of CD19+ B cells from CD40LΔ5 mice. (A) CD19+ B cells were isolated from WT and CD40LΔ5 mice 8 d after immunization with SRBC. Hierarchical clustering of gene expression profiles using differentially expressed genes (>1.5-fold) between groups (normalized log2 values based on RNA-sequencing analysis) is shown above. Genes with a statistically significant false discovery rate (q < 0.05) are displayed and grouped by function (n = 3 biological replicates). (B) GO pathways showing statistically significant changes in B cells from CD40LΔ5 mice. (C) Analysis of intracellular expression of Ki67 (left panel) and quantification of results (right panel) of splenic GL7+ B cells 8 d following SRBC injection. (D) Graphical representation of intracellular expression of phospho-histone H3 (PHH3) in GL7+ B cells 8 d following injection with SRBCs (left panel) and quantification of n = 4 independent experiments (right panel). (E) Flow cytometric image of intracellular expression of activated Caspase-3 (y-axis) and cleaved PARP (x-axis) in GL7+ cells from WT and CD40LΔ5 mice (left panels) and compiled results for four independent experiments (right graphs). Each symbol represents an individual mouse and the mean and SEM are indicated by horizontal bars. Significance was determined by unpaired, two-tailed t test with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
higher level of ongoing apoptosis in these cells (Fig. 4E). Together, these results reveal that the elevated expression level of CD40L resulting from increased mRNA stability is required for optimal proliferation and cell survival of both CD19+ B cells and GC B cells following immunization. Therefore, changes in both proliferation and apoptosis likely underlie the loss of GC B cells in CD40LΔ5 mice.

Loss of the CD40L mRNA stability pathway leads to a reduction in splenic Ab-secreting B cells

Because CD40L is critical for both early and late Ab responses to TD Ags, we first examined whether the CD40L mRNA stability pathway was critical for early Ab responses that may occur in part, outside the GC (1, 4, 20, 48, 49). CD40LΔ5 and WT mice were immunized with NP-KLH and blood collected at 1, 2, and 3 wk. Anti-NP Abs were evaluated for IgM and for the presence of switched Abs of the IgG1, IgG2b, and IgG2c subclasses. Whereas we observed no obvious decrease in IgM Abs, the levels of IgG1, IgG2b, and IgG2c were significantly lower in mutant compared to WT mice (Fig. 5A). Analysis of the secondary response in animals at 28 d after immunization and 7 d after boosting revealed a significant decline in humoral output in CD40LΔ5 mice including a significant decrease in IgM, IgG1, IgG2b, and IgG2c Abs. In contrast, there was no observable difference in IgG3 and IgA although the overall level of IgA was very low in the serum (Fig. 5B).

We next used ELISpot to determine if the basis for the loss of TD Abs in CD40LΔ5 mice was because of decreased numbers of responding B cells or alternatively corresponded to lowered Ab expression per responding cell. IgG1-specific ASCs were harvested from spleen at day 28 following injection of NP-KLH at days 0 and 21. Notably, over a wide range of dilutions there was a clear decrease in the number of ASCs in CD40LΔ5 mice indicating that the CD40L stability pathway resulted in the reduction of ASCs and not in a lowered amount of Ab produced per cell (Fig. 5C).

The CD40L mRNA stability pathway is required for optimal affinity maturation but not SHM

To determine whether SHM and affinity maturation were linked to the higher threshold of CD40L expression that resulted from mRNA stability, experiments were carried out to identify nucleotide changes in the VH regions that are known to be associated with increased Ab affinity. In the NP system, early replacement
of a tryptophan (W) with a leucine (L) at codon 33 in the VH186.2 gene has been shown to be associated with a 10-fold increase in affinity of anti-NP Abs (50, 51). Therefore, RNA was isolated from PNA + splenocytes 14 d after immunization with NP-KLH and corresponding cDNAs sequenced across the VH region. We observed that 40% of the CD40LΔS sequences had the G → T transversion leading to the W33L substitution compared with 60% of the WT sequences (Fig. 6A). Although this difference did not reach statistical significance, the downward trend prompted us to ask whether there was a corresponding decrease in the number of mutations across the CD40LΔS VH186.2 gene, which would indicate a potential acquired immunodeficiency. Surprisingly, we found no consistent difference in the total number of mutations in B cells from CD40LΔS and WT mice suggesting that the lower level of CD40L does not appreciably impact SHM (Fig. 6B). To further confirm these findings, GC B cells were analyzed for expression of AICDA and c-myc, which have both been shown to be controlled, in part, by CD40 signals (52, 53). Notably, we observed a highly significant decrease in c-myc expression and no difference in AICDA expression in PNA + B cells from CD40LΔS mice (Fig. 6C).

Because there was no apparent effect of a lowered threshold of CD40L expression on SHM we next asked whether there was a general loss of affinity-matured Abs relative to total Abs. CD40LΔS and WT mice were immunized with NP-KLH and analyzed by ELISA at four time points for the production of anti-NP Abs. NP-BSA conjugates with a low or high number of haptens per BSA molecule were used as Ags to establish the degree of ongoing affinity maturation (54, 55). Similar to our findings that examined the total spectrum of Ab responses (Fig. 5A, 5B) the overall high-affinity IgG1 Ab response was significantly reduced in the CD40LΔS mice. However, the ratio of high-affinity to total Ab was not significantly different (Fig. 6D, 6E). Therefore, the higher threshold of CD40L expression is critical for the expansion of the high-affinity B cells in the GC but is not absolutely required for SHM or for affinity maturation.

**The CD40L stability pathway is required for the optimal development of plasma cells**

We next asked whether the CD40L stability pathway also impacted downstream fate decisions that resulted in the differentiation of functional B cell subpopulations. We first analyzed the B220+ ASCs that re-express CD138 upon differentiation into plasmablasts following SRBC injection. As predicted by the loss of GC B cells in CD40LΔS mice, we observed a significant decline in the absolute number of B220+CD138 + cells (plasmablasts) as well as a decrease in the frequency of plasmablasts within the B220 + population (Fig. 7A). We extended this analysis to compare numbers and percentages of both IgM and IgG1 plasmablasts that expressed CD138 and were positive for intracellular Ig. As shown in Fig. 7B and 7C, an approximate 3-fold reduction in both the total number and percentage of IgM plasmablasts was observed in CD40LΔS mice. Further analysis of the B220 + IgM + cells for expression of intracellular IgG1 and surface CD138 identified a significant decline in the IgG1-expressing plasmablasts in both total number and percentage (Fig. 7D). Finally, to determine whether observed differences in splenic populations were maintained in the long-lived plasma cells (LLPCs) resident in the BM (B220 + CD93 + CD138 +), mice were immunized with SRBC and BM cells harvested. Similar to our findings with splenic plasma cell subsets, both the number (1.0 × 10^5 versus 2.5 × 10^4) and percentage (0.8% versus 3.3%) of LLPCs relative to total BM B220 + cells were decreased in CD40LΔS mice relative to WT controls, respectively (Fig. 7E).

Altered CD40L expression has a disproportionate impact on memory B cells

To investigate the role of the CD40L stability pathway on the development of (CD19 + CD38 + IgG1 +) precursor memory B cells, mice
FIGURE 7. Decreased number and percentage of plasma cell subsets in the CD40LΔ5 mice. (A) Representative flow cytometric analysis of B220⁺CD138⁺ plasma cells from WT and CD40LΔ5 spleens, 8 d after SRBC immunization and graphical representation of compiled data from individual mice showing the total number and percentage of plasma cells (right graphs). (B) Gating scheme for splenic subsets shown in (C) and (D). (B) Representative flow cytometry (left panels) and compiled data (right graphs) of the total number and frequency of B220⁺CD138⁺IgM⁺ cells (Figure legend continues).
were analyzed after SRBC injection for both the number and percentage of switched IgG1+ B cells. Similar to our finding with plasma cells there was a significant decrease overall in precursor memory B cells from CD40LΔS mice (Fig. 8A, 8B). Given that memory B cells can include different isotypes including IgM+ B cells (56–59), we sought to determine whether differences were observed in switched and unswitched subpopulations from CD40LΔS and WT mice. Additionally, we wanted to assess expression of PD-L2, CD80, and CD73, three surface markers, which define developmental and functional heterogeneous populations within the memory B cell compartment (59, 60). We found a decrease in all PD-L2, CD80, and CD73 precursor subsets defined by either IgG1 or IgM from mice expressing the CD40LΔS mutation (Fig. 8D, 8F, upper graphs). The overall decrease in numbers of all precursor subsets was consistent with the loss of GC B cells in the CD40LΔS mice. However, the percentage loss of IgG1+ and IgM+ B cells was not anticipated, and this loss was only seen in the precursor subsets defined by markers CD80 or CD73, and not PD-L2 (Fig. 8D, 8F, lower graphs). The striking decline in precursor memory B cells of both IgM and IgG1 subsets, strongly suggests that a threshold of CD40L expression is critical for the development of memory B cells regardless of whether the cell has undergone class switching. These results also indicate that cells that fail to enter a plasma cell developmental pathway are not necessarily shunted into a memory pathway and thus, the differentiation of both B cell effector subpopulations appears to be exquisitely sensitive to a higher threshold of CD40L that depends on mRNA stability.

Discussion

It has long been known that CD40 signaling is central to the development of a GC response and the multiple studies that analyzed deficiencies in either CD40 or CD40L established the groundwork for understanding the mechanistic basis for the development of humoral immunity (61). In this study, we sought to extend the binary understanding of CD40 signaling by using a mouse model that provides reduced levels of CD40L through a change in mRNA stabilization. The model was predicated on previous work showing that the CD40L mRNA is stabilized through the binding of PTBP1-containing complexes to a region of the 3′UTR (27, 36, 37). These complexes include other RNA-binding proteins such as nucleolin, and other potentially unidentified proteins. Notably, we found that the optimization of many primary and secondary TD B cell responses relies on a threshold level of T cell CD40L that is directly linked to enhanced mRNA stability. Whereas the elimination of the stability pathway results in a reduction of CD40L expression on TFH cells by ~40%, the overall impact of this decrease on Ab output and the development of effector B cell subsets is considerably more pronounced. Our data strongly suggest that this disproportionate response corresponds to the combined effects of limited cellular expansion linked to proliferation defects in Ag-selected B cells, increased apoptosis in GC B cells as well as reduced differentiation of GL7+ B cells into plasmablasts and memory precursor cells.

Although many of the cell populations and effector molecules in CD40LΔS mice were greatly affected by the decrease in CD40L expression, we also found little to no effect on other functional processes. For example, primary IgM and secondary IgG3 Ab levels were similar between CD40LΔS and WT mice, which is consistent with results showing that patients with hyper-IgM syndrome mount IgM responses to TD Ags and mice lacking CD40L express both IgM and IgG3 within a normal range (61, 62). Also, class switch recombination (CSR) was intact in B cells from CD40LΔS mice although the serum titer of primary and secondary IgG1, IgG2a, -2b, and -2c and the secondary IgM Abs was significantly lower because of fewer numbers of ASC in the B cell pool. The fact that CD40LΔS mice were capable of undergoing CSR is consistent with reports showing that this process occurs primarily as extrafollicular events prior to B cell seeding of the GC (63). Thus, the disruption in the GC architecture observed in CD40LΔS B cells appears to have little impact on class switching, per se, and the decline in the number of switched cells is likely associated with a loss of proliferative capacity via interactions with CD4 T cells expressing lower levels of CD40L in the extrafollicular space. It has yet to be determined whether the severe loss of ASCs is a byproduct of decreased CD40 signaling needed to drive a specific number of cell divisions linked to class switching and/or the requirement for these signals in the subsequent clonal expansion of the isotype switched B cells (64, 65).

We also found that both SHM and affinity maturation were relatively normal in B cells from CD40LΔS mice although the absolute number of cells expressing high-affinity mutations in their BCRs was reduced. This is consistent with SHM occurring in association with proliferation in the DZ and reflects the severe loss of resident GL7+ B cells in this compartment. The finding of WT levels of somatically mutated sequences in GC B cells from CD40LΔS mice is further supported by our observation that the level of Aicda RNA in these cells was similar to WT cells (66, 67). It may be that SHM can occur in DZ B cells in response to the lower threshold of CD40-CD40L signals received by the LZ B cells or alternatively SHM is able to function in the absence of CD40 signaling altogether. Support for this second possibility comes from studies demonstrating that B cells from patients with hyper-IgM syndrome undergo SHM via a CD40-CD40L-independent pathway (68, 69).

T cell help is considered to be the limiting factor in GC B cell selection and TFH cells have been shown to express a comparatively low level of CD40L (18, 19, 70). Furthermore, enhanced TFH interactions with LZ B cells accelerates S phase and directly corresponds to the number of cell divisions and mutations that will occur during a single-selection cycle (19). The proto-oncogene c-myc is essential for GC B cells to efficiently integrate CD40 signals leading to functional outcomes, including positive selection and cyclic re-entry of LZ B cells (52, 53, 71). Importantly, c-myc expression in GC B cells is tightly controlled by CD40L expression on TFH cells and the absolute amount of MYC expressed in GC LZ B cells determines the subsequent number of cell divisions in the DZ (72). Our data showing that the diminished population of GC B cells from CD40LΔS mice express only 25% of the c-myc RNA levels found in WT cells agrees with these findings. Finally, the loss of GL7+ B cells in the DZ may also be explained, in part,
FIGURE 8. CD40LΔ5 mice have a significantly lower percentage of splenic prememory cells. (A) Representative flow cytometric analysis of IgG1+CD38+CD19+ precursor memory cells from WT and CD40LΔ5 spleens, 8 d after SRBC immunization and (B) graphical representation of individual mice showing the total cell counts and the percentage of CD19+ B cells. (C) Representative flow cytometry analysis of WT (top row) and CD40LΔ5 (bottom row) splenic IgM B cell precursor memory subsets expressing markers PD-L2, CD80 and CD73. (D) Graphical representation of total cell counts (top graph) and the frequency of each subgroup (bottom graph). (E) CD19+IgM+IgG1− cells evaluated for expression of PD-L2, CD80, and CD73 (n = 4), and (F) data presented as total counts and frequency. In all graphs, center values denote the mean, and error bars denote SEM. Determination of significance was carried out by unpaired, two-tailed t test where **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. NS, not significant.
by enhanced apoptosis in both regions of the GC given that apoptosis has been shown to be the “default” pathway for GC B cells (73, 74).

The impact of decreased CD40L expression was observed on cell fate decisions within the GC affecting both the plasmablast and precursor memory B cell subsets. Our results showing that the percentage of B cells expressing memory markers was significantly reduced in CD40LKO mice was highly unexpected given the fact that B memory cells have lower numbers of mutations in their BCRs. Additionally, B memory cells were found to exit the GC response at earlier iterations compared with their plasma cell counterparts (75). We anticipated, given the decreased cycling rate and reduced response at earlier iterations compared with their plasma cell counterparts, that B memory cells have lower numbers of mutations in their BCRs (76). Also, reduced cytokine production such as IL-4 and IL-21, which are known to be required for driving the differentiation of GC B cells (76). Also, reduced cytokine production such as IL-4 and IL-21, which are known to be required for driving the differentiation of GC B cells (76).

Alternatively, reducing the level of CD40 signaling may impact other critical factors such as IL-4 and IL-21, which are known to be required for driving the differentiation of GC B cells (76). Also, reduced cytokine production such as IL-4 and IL-21, which are known to be required for driving the differentiation of GC B cells (76). Also, reduced cytokine production such as IL-4 and IL-21, which are known to be required for driving the differentiation of GC B cells (76).

We thank Dr. Derek Sant’Angelo for critically reading this manuscript and for many helpful discussions and insight. We acknowledge Dr. Mike Kaledjian as well as past and present laboratory members of the Covey laboratory for valuable contributions to this project. pEasyl Flox was a gift from Klaus Rajewsky.

Acknowledgments

The authors have no financial conflicts of interest.

Disclosures

References


Supplementary Information for:
A Posttranscriptional Pathway of CD40L mRNA Stability is required for the Development of an Optimal Humoral Immune Response

Bitha Narayanan, Diego Prado de Maio, James La Porta, Yekaterina Voskoboynik, Usha Ganapathi, Ping Xie and Lori R. Covey
Supplementary Figure 1. (a) Schematic of the mCD40L cDNA showing the coding region, 3'UTR stability element and end of exon 5 (1736). (b) Luciferase assays carried out with regions of the CD40L stability element cloned into the 3’ region of the pRL-SV40 vector. Results are normalized to expression of Renilla luciferase. (c) Schematic diagrams of the CD40L gene showing targeted regions (exons 4 and 5), CD40L Δ5 construct, the targeted locus with the NeoR gene indicated, and the targeted locus after Cre-mediated recombination. Indicated in red is the CD40L translational stop codon and polyA site (114 nt from loxp site). Location and size of the three CD40L sequences used in the targeting vector, the neoR and TK cassettes, and loxP sites are indicated. PCR primers for screening ES cell clones for homologous recombination are depicted with arrows. Position of probe to analyze Southern blots is indicated as probe A. (d) ES cell clones analyzed for proper recombination into the genomic locus; clones 84 and 89 of this analysis were used to generate lines of CD40LΔ5 mice. (e) Representative PCR from mice generated from clone 84 and carried out with neoF-6567R (right flank) and 7505F-neoR (left flank) primers from outside of the targeting construct. (f) Representative PCR analysis of genomic DNA from mice following deletion of the NeoR gene showing hemizygous male mutants (y/Δ5), heterozygous (+/Δ5), homozygous (Δ5/Δ5) and WT females using primers Δ5F and Δ5R.
Supplementary Figure 2. Evaluation of T and B cell populations in CD40LΔ5 mice. (a) Thymus and spleens were collected from 6-10-week-old mice, dissociated and analyzed for distribution of B and T cells. Thymus (top panels) and CD3-gated spleen (bottom panels) were analyzed for CD4 and CD8 expression. Histograms (right) showing the Mean+/−s.e.m. from n=3 experiments. (b) Spleens were collected from naïve (left 2 panels) and day 28 NP-KLH immunized mice (boosted at day 21, right 2 panels). Splenic MZ (IgM^{hi}, IgD^{lo}) and FO (IgM^{lo}, IgD^{hi}) are boxed and bar graphs of 4 independent experiments are presented on the right. (c) Representative image shows splenic MZ (B220^{+}, CD23^{lo}/CD21^{hi}), and FO (B220^{+},CD23^{hi}/CD21^{lo}) populations in WT and CD40LΔ5 mice and data from 3 independent experiments compiled in graphs on the right. Significance was determined using student’s unpaired t test where a p>0.05 is considered “not significant” (NS).
Supplementary Table 1:
Antibodies used for Flow cytometry and Immunohistochemistry. Antibodies designated in **bold** were used in in IHC.

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### Supplementary Table 2.

**Primers used to generate and analyze the CD40LΔ5 construct**

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<td>V186.2-leader</td>
<td>J00529</td>
<td>5'-agcgtctagatcgcttttgcc-3'</td>
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<td>MH397224.1</td>
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