

## Humoral Immune Responses in CD40 Ligand-deficient Mice

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### Summary

Individuals with X-linked hyper-IgM syndrome fail to express functional CD40 ligand (CD40L) and, as a consequence, are incapable of mounting protective antibody responses to opportunistic bacterial infections. To address the role of CD40L in humoral immunity, we created, through homologous recombination, mice deficient in CD40L expression. These mice exhibited no gross developmental deficiencies or health abnormalities and contained normal percentages of B and T cell subpopulations. CD40L-deficient mice did display selective deficiencies in humoral immunity; basal serum isotype levels were significantly lower than observed in normal mice, and IgE was undetectable. Furthermore, the CD40L-deficient mice failed to mount secondary antigen-specific responses to immunization with a thymus-dependent antigen, trinitrophenol-conjugated keyhole limpet hemocyanin (TNP-KLH). By contrast, the CD40L-deficient mice produced antigen-specific antibody of all isotypes except IgE in response to the thymus-independent antigen, DNP-Ficoll. These results underscore the requirement of CD40L for T cell-dependent antibody responses. Moreover, Ig class switching to isotypes other than IgE can occur *in vivo* in the absence of CD40L, supporting the notion that alternative B cell signaling pathways regulate responses to thymus-independent antigens.

Antigen (Ag)-specific activation of B cells occurs in two distinct steps. In the first, Ag binds to membrane Ig on the B cell surface, is degraded and processed, and is then presented on the cell surface in association with MHC class II molecules (1, 2). The second step involves the recognition of the Ag-MHC class II complex by T cells that become activated and deliver a stimulatory signal back to the B cells (3, 4). Whereas Ag recognition by T cells is MHC-restricted, the subsequent signal delivered by the helper T cell to the Ag-presenting B cell is both Ag-independent and genetically unrestricted (5–8). Recently, the ligand for CD40 (CD40L)<sup>1</sup> has been identified as a major component of contact-dependent T cell-mediated B cell activation (9–11).

CD40L is a 33-kD glycoprotein that is transiently expressed on the surface of activated T cells, predominantly of the CD4<sup>+</sup> phenotype (10, 12, 13). CD40L provides a mitogenic signal to B cells, the potency of which is enhanced by soluble cytokines, notably IL-4 and IL-5 in the mouse (14) and IL-4 and IL-10 in the human (15, 16). These same cytokines to-

gether with CD40L also provide costimulatory signals that induce secretion of polyclonal Ig of multiple isotypes (14–17). The process of B cell affinity maturation and isotype switching after antigenic challenge requires CD40L. Defects in the CD40L gene result in X-linked hyper-IgM syndrome (HIGM), a condition characterized by elevated serum concentrations of IgM with a virtual absence of other Ig isotypes, an increased susceptibility to opportunistic bacterial infections, and an absence of germinal centers in secondary lymphoid tissue. Experimental evidence for the role of CD40L in the generation of Ag-specific B cell responses came from studies that demonstrated that *in vivo* administration of a neutralizing antibody specific for murine CD40L inhibited both the primary and secondary Ag-specific humoral response to thymus-dependent (TD) antigens (18). In addition to its activities on B cells, CD40L has stimulatory effects on monocytes, inducing cytokine secretion and tumoricidal activity (19), and on T cells where it costimulates proliferation and cytokine secretion and induces the generation of cytolytic T cells (20–22).

To better understand the requirements for CD40L function in an *in vivo* context, we have generated mice containing a disrupted CD40L gene. We demonstrate in this report that these CD40L “knockout mice” (CD40LKO) are unable to

<sup>1</sup> Abbreviations used in this paper: BGG, bovine  $\gamma$  globulin; CD40L, CD40 ligand; ES, embryonic stem; HIGM, hyper-IgM syndrome; HRP, horseradish peroxidase; KO, knockout; TD, thymus-dependent; TI, thymus-independent; TNP, trinitrophenol.

mount an Ag-specific humoral response to a TD Ag and fail to develop germinal centers in secondary lymphoid tissue after antigenic challenge. These mice can, however, respond to a thymus-independent (TI) Ag as demonstrated by their ability to generate Ag-specific Ig of all isotypes except IgE. The results suggest that the CD40LKO mouse will be a useful model for further studies on the role of CD40L and other cell surface molecules in various aspects of the immune response.

## Materials and Methods

**Animals.** Experiments were performed with C57BL/6 (Charles River Laboratories, Wilmington, MA) or Swiss Webster (Taconic Farms Inc., Germantown, NY) mice. Animals were maintained under specific pathogen-free conditions.

**Reagents.** The following reagents were used for *in vitro* B cell assays: recombinant murine IL-4 (Immunex Corp.) and IL-5 (R&D Systems Inc., Minneapolis, MN); *Salmonella typhimurium* LPS (Difco Laboratories, Detroit, MI); affinity-purified goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL); recombinant murine soluble CD40L (Immunex). CD40L was purified from conditioned medium of Chinese hamster ovary (CHO) cells stably expressing a cDNA plasmid encoding a fusion protein consisting of CD40L (extracellular region) and a modified leucine zipper motif, resulting in secretion of a soluble, trimeric form of CD40L (23). The following ELISA reagents were employed: unconjugated and horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-mouse IgM, IgA, IgG1, IgG2b, and IgG3 (Southern Biotechnology Associates); and unconjugated and biotinylated rat anti-mouse IgE (Bioproducts for Science, Indianapolis, IN).

Conjugated antibody reagents were used to detect surface expression of hematopoietic cell markers. Surface IgD<sup>+</sup> and surface IgM<sup>+</sup> B cells were detected using biotin-labeled mouse anti-IgD<sup>b</sup>-clone 217-170 (PharMingen, San Diego, CA) and goat (F(ab')<sub>2</sub>) anti-mouse-IgM-FITC conjugate (Tago, Inc., Burlingame, CA), respectively. B cells were also monitored using CD45R-FITC (PharMingen). T cell subsets were analyzed using CD3-FITC (22), CD4-FITC and CD8-FITC (Caltag Laboratories, San Francisco, CA). CD11b-biotin (PharMingen) was used to detect macrophages. Mouse IgG1-FITC and IgG1-PE-conjugated control mAb were obtained from Becton Dickinson & Co. (Mountain View, CA).

**Gene Targeting Vector Construction.** All DNA manipulations were performed using standard methodology (24). To facilitate construction of a targeting vector, a murine (129/SV strain)  $\lambda$  genomic library (Stratagene, La Jolla, CA) was screened using a radiolabeled CD40L cDNA probe based upon the 800 bp SalI-PstI fragment of murine CD40L (12). Two clones were subsequently restriction mapped and used to construct the targeting vector. pGEM11 (Promega Corp., Madison, WI) was used as the cloning vector throughout. pHRV-mCD40L was constructed by inserting a 1.3-kb XbaI-SpeI genomic fragment (located ~800 bp downstream of exon 2) 5' of the neomycin phosphotransferase gene driven by the murine PGK-1 promoter (PGK/Neo) (25). A 12.2-kb HindIII-SalI genomic fragment (containing exon 5) followed by the HSV-TK gene (26) was inserted immediately 3' of PGK/Neo. The replacement vector was designed to delete exons 3 and 4 after homologous recombination.

**Electroporation, Selection, and Analysis of Embryonic Stem (ES) Cells.** After linearization with NotI, pHRV-mCD40L was electroporated into the ES cell line D3 (27) at 200 V and 960  $\mu$ F. Cells were cultured on a feeder layer of  $\gamma$ -irradiated, neomycin-resistant STO cells expressing LIF and were selected in 175  $\mu$ g/ml G418 (GIBCO BRL,

Gaithersburg, MD) plus 2 mM gancyclovir (Syntex, Palo Alto, CA) for ~10 d. After selection, plates were washed once with PBS and flooded with trypsin. After 2 min, the trypsin was removed and replaced with serum-containing medium. Individual colonies were isolated in 100  $\mu$ l media and dispersed by repeated pipetting in a 96-well plate. Clones were then plated singly and analyzed via PCR in pools of five clones.

Targeted ES clones were identified by PCR amplification using an antisense primer specific for the PGK-1 promoter (P1: 5'-CTTGTTAGCGCCAAGTG-3') in conjunction with a CD40L intronic primer that lies immediately upstream of the 5' terminus of pHRV-mCD40L (P2: 5'-GTATGTGGCTGAACACCTG-3'). PCR analysis was performed in 100  $\mu$ l reaction volumes in 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-Cl, pH 8.4, 200  $\mu$ M each dNTP, 25 pmol each primer, and 2.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). 30 cycles were performed at 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min. After amplification, 20  $\mu$ l of each reaction were separated by electrophoresis on 1% agarose gels, and product was visually observed after staining with ethidium bromide. The presence of a 1.6-kb product indicated homologous recombination.

Homologous recombination in PCR-positive clones was confirmed by genomic Southern blot analysis. High molecular weight DNA (15  $\mu$ g) was digested with PstI, electrophoresed through 1% agarose, blotted to nitrocellulose, and probed with a radiolabeled 400-bp XbaI-PstI genomic fragment located upstream of the 5' terminus of pHRV-mCD40L. This probe hybridized to a 9.0-kb PstI fragment in wild-type genomes, while gene targeting introduced a new PstI site 2.2 kb downstream of the 5' PstI site.

**Generation of CD40L-deficient Mice.** Cells from one clone, no. 9-72, were injected into day 3.5 blastocysts isolated from C57BL/6 mice and carried to term in pseudopregnant Swiss Webster females. Resultant male chimeras were subsequently mated with wild-type C57BL/6 females to derive females heterozygous for the targeted mutation. [129/SV  $\times$  C57BL/6] F<sub>1</sub> heterozygous females (+/-) were then crossed with C57BL/6 males to derive the hemizygous (-/0) and wild-type (+/0) male animals analyzed in the present study.

Progeny from the above matings were genotyped via four-primer PCR of ear biopsy DNA extracts. Two primers specific for the region deleted in targeted mutants (P3: 5'-CCCAAGTGTATG-AGCATGTGTGT-3' and P4: 5'-GTTCTCCACCTAGTCATTCATC-3') amplify a 250 bp product from the wild-type allele only. These were used in conjunction with two primers specific for PGK/Neo (P5: 5'-GCCCTGAATGAACAGTGCAGGACG-3' and P6: 5'-CACGGGTAGCCAACGCTATGTC-3') that amplify a 500-bp band in the mutant allele only. 35 cycles were performed at 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. Representative progeny were also analyzed by genomic Southern blotting as described above.

**Histochemical Analysis.** Spleen or lymph node (LN) specimens were coded and examined in a blinded fashion. For histological studies, tissue samples of the spleen and LNs were fixed in Methocarbonyl's fixative (28) for 1 h at room temperature. Further tissue processing was performed on a tissue processor (V.I.P., Miles Laboratories, Inc., Naperville, IL) as follows: dehydration in 100% alcohol, clearing in Shandon's Xylene substitute (Shandon, Inc., Pittsburgh, PA), and infiltration in Tissue Prep paraffin. Specimens were then embedded in Tissue Prep 2 paraffin (Fisher Scientific, Fairlawn, NJ), sectioned at 3  $\mu$ M and stained with hematoxylin and eosin.

**Flow Cytometric Analysis of Cell Surface Ag Expression.** Lymphoid organs were harvested aseptically and cell suspensions prepared from LN and spleen and resuspended in PBS containing 1% FCS, 10% normal goat serum and 0.1% NaN<sub>3</sub> before cytofluoro-

metric analysis. Antibody or Fc fusion protein binding (CD40.Fc and IL-4R.Fc) and one- or two-color flow cytometric analysis was performed as described (12).

### Immunization Protocols

**TD Ag.** Trinitrophenol-conjugated keyhole limpet hemocyanin (TNP-KLH) was precipitated with alum under pyrogen-free conditions. On day 0 of the immunization protocol, mice were injected intraperitoneally with 1  $\mu$ g of TNP-KLH in a volume of 200  $\mu$ l. Identical secondary immunizations were administered on day 21. Mice were bled on day 0 and on day 26 and serum was prepared for ELISA analysis.

**TI-2 Ag.** On day 0 of the immunization protocol, mice were injected intraperitoneally with 10  $\mu$ g (100  $\mu$ l) of DNP-Ficoll, generously provided by Dr. Bondada Subbarao (University of Kentucky, Lexington, KY). On day 10, mice were bled and serum was prepared for ELISA analysis.

**Determination of Serum Polyclonal and Anti-TNP Ig Levels.** Serum polyclonal IgA, IgE, IgG1, IgG2b, IgG3, and IgM levels were determined by an isotype-specific ELISA technique as previously described (29). Briefly, individual wells of 96-well plates (Flow Laboratories Inc., McLean, VA) were coated with anti-mouse isotype-specific antibody and blocked with 5% nonfat dry milk in PBS. Serially diluted serum samples were added, followed by the appropriate HRP-conjugated second-step antibody. For the IgE isotype specific assay, biotinylated rat anti-mouse IgE was used as second antibody reagent and HRP-conjugated streptavidin (Zymed Laboratories, Inc., S. San Francisco, CA) was used in the following step. All assays were developed using the TMB Microwell peroxidase substrate system (Kierkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Ig concentrations in serum test samples were determined by comparing test sample dilution series values with isotype control standard curves using the DeltaSoft 2.1 ELISA analysis program (Biometallics, Inc., Princeton, NJ). Where indicated in Results, Student's *t* test (for two samples assuming unequal variance) was used to generate *p* values.

Anti-TNP Ig isotype levels were determined by a modified sandwich ELISA method (30). Plates were coated with goat anti-Ig isotype, blocked with nonfat dry milk, and incubated with serially diluted test serum samples. Biotinylated TNP-bovine  $\gamma$  globulin (TNP-BGG) was used as a third step followed by HRP-conjugated streptavidin. Wells were developed with peroxidase substrate and analyzed as described above. Multiple point analysis was performed on each set of isotype titrations using the BIOASSAY program, selecting a maximum value for each isotype and determining for each sample the dilution giving half-maximal OD value, thus generating arbitrary U/ml values as previously described (30).

**Splenic B Cell Culture.** B lymphocytes were purified from spleens as previously described (31) by a combination of T cell depletion with antibody plus C', adherent cell depletion over Sephadex G10 columns, and positive selection on anti-IgM coated pans. The resultant preparations were >99% pure B cells as determined by flow cytometric analysis for surface IgM expression. B cells were cultured in RPMI 1640 supplemented with 5% FCS (JRH Biosciences, Lenexa, KS), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), L-glutamine (2 mM), and 2-mercaptoethanol (50  $\mu$ M). For proliferation assays, B cells ( $10^5$ /well; 200  $\mu$ l) were cultured in 96-well flat-bottom tissue culture plates (Costar Corp., Cambridge, MA) with or without 10 ng/ml each of IL-4 and IL-5 either alone or in the presence of LPS, goat anti-mouse IgM, or purified recombinant soluble CD40L. Cultures were pulsed for the last 16 h of a 72-h

culture period with 2  $\mu$ Ci/well of [ $^3$ H]thymidine (25 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 16 h. Cells were then harvested onto glass fiber filters, and incorporation of radioactivity was measured by tritium sensitive avalanche gas ionization detection on a Matrix 96 Direct Beta Counter (Packard Instruments, Meriden, CT).

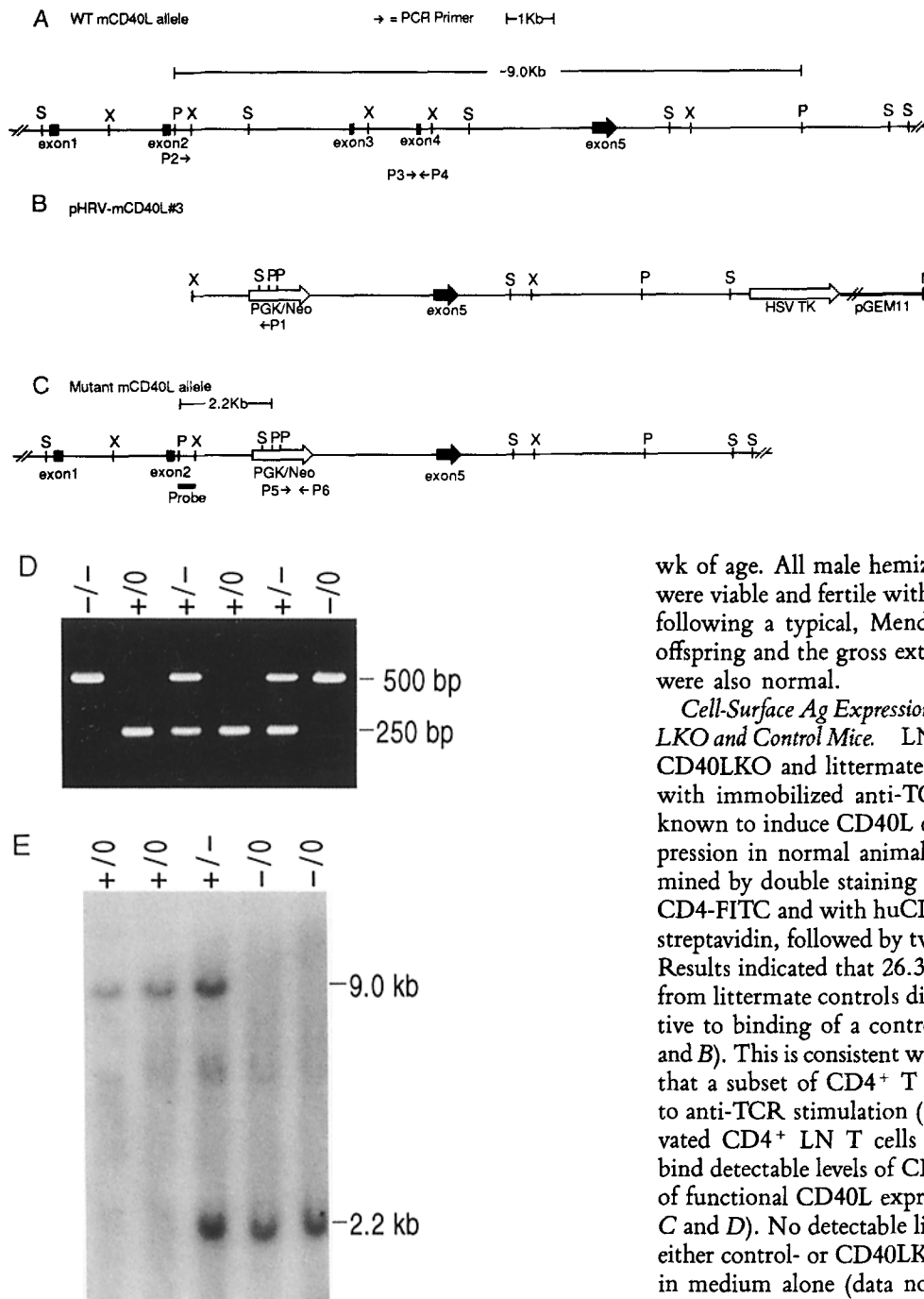
For polyclonal Ig secretion, B cells ( $5 \times 10^5$  cells/well) were grown in 96-well flat-bottomed plates for 6 d either alone or in the presence of IL-4 plus IL-5 (each at 10 ng/ml) plus recombinant soluble CD40L or LPS. Cells were then pelleted by centrifugation at 750 *g*, and culture supernatant fluids were harvested for quantification of Ig levels by isotype-specific sandwich ELISA as described above.

### Results

**Generation of CD40L-deficient Mice.** To generate a null mutation in the murine CD40L gene (Fig. 1 A), a targeting vector employing a standard positive-negative selection (26) was constructed. pHRV-mCD40L (Fig. 1 B) contains 13.5 kb of CD40L genomic sequences in which the positive selection marker, PGK/Neo (25) is flanked by a 12.2-kb HindIII-SalI genomic fragment (containing exon 5) on the 3' side, and a 1.3-kb XbaI-SpeI genomic fragment (located 800 bp 3' of exon 2) on the 5' side. The HSV TK gene (26) was added 3' of the 12.2-kb stretch of homology to select against random integration events. The vector was designed to delete exons 3 and 4 of CD40L after homologous recombination at the endogenous gene (Fig. 1 C).

ES cells of the 129/SV-derived line D3 (27) were electroporated with NotI-linearized vector and selected in the presence of G418 and gancyclovir. 80 colonies surviving double selection were screened for targeting events by PCR using a primer specific for the endogenous gene (Fig. 1 A) and an antisense primer specific for PGK/Neo (Fig. 1 B). The presence of a 1.6-kb product was indicative of homologous recombination and was observed after amplification of two clones. Thus the frequency of homologous recombination was 1 in 40 doubly resistant clones. Gene targeting was confirmed by genomic Southern blot analysis.

One targeted ES clone, no. 9-72, was injected into C57BL/6 blastocysts which were then transferred into pseudopregnant recipient females (Swiss Webster) to generate chimeras. Germ-line transmission and subsequent segregation of the mutant allele was monitored by PCR amplification using four primers simultaneously (Fig. 1 D). Two primers (P3 and P4) define a 250-bp product within the region deleted by gene targeting and amplify exclusively the wild-type allele. The other two primers (P5 and P6) are specific for PGK/Neo and amplify only a 500-bp product from the mutant allele. The genotypes of representative animals were further analyzed by genomic Southern blotting (Fig. 1 E). The radiolabeled probe hybridized to a 9.0-kb PstI-generated band in wild-type genomes. Gene targeting, however, introduced a new PstI site downstream of the 5' PstI site, resulting in a 2.2-kb band (Fig. 1 E). [129/SV  $\times$  C57BL/6] $F_1$  female heterozygotes (+/-) were crossed to wild-type C57BL/6 males to yield the hemizygous (-/0) and wild-type (+/0) male animals analyzed in the present work. Experimental animals were all 6-8-



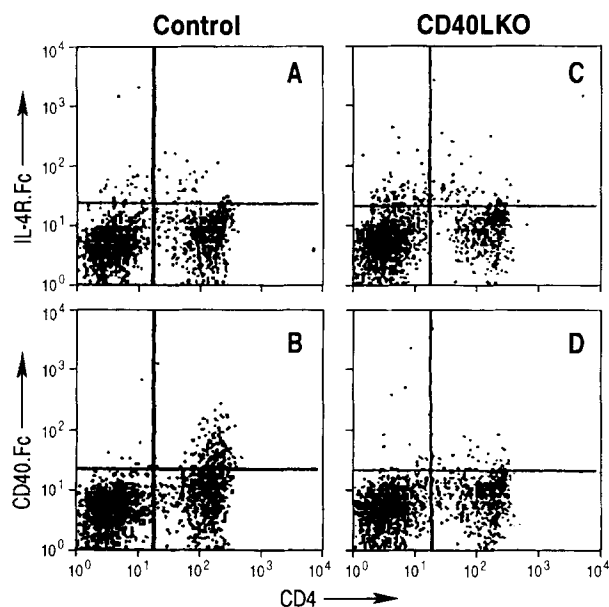
**Figure 1.** Targeted mutation of the X-linked murine CD40L gene. (A) Schematic diagram of the wild-type allele. Exons are represented as *thick, black boxes*. (S) SpeI; (X) XbaI; (P) PstI; (N) NotI. P3 and P4 are PCR primers specific to the region deleted in the targeted allele. (B) Gene targeting vector, pHRV-mCD40L. P1 represents the PCR primer used with P2 (endogenous gene primer) to detect homologous recombination events. (C) Structure of the mutant CD40L allele. P5 and P6 are neomycin phosphotransferase gene PCR primers unique to the mutant allele. The 400-bp XbaI-PstI fragment located just downstream of exon 2 was used as the probe in (D). (D) Genotype analysis of ear biopsy DNA. Heterozygous females were crossed with wild-type male C57BL/6 mice. DNA extracted from ear biopsy samples were coamplified with primers P3, P4, P5, and P6. Primers P5 and P6 amplify a 500-bp product from the mutant allele only, and P3 and P4 define a 250-bp amplification product specific to the

wild-type allele. All male hemizygotes and female homozygotes were viable and fertile with segregation of the mutant allele following a typical, Mendelian pattern. Sex ratios among offspring and the gross external phenotype of these animals were also normal.

**Cell-Surface Ag Expression and CD40L Expression in CD40LKO and Control Mice.** LN T cells were obtained from both CD40LKO and littermate control mice and then activated with immobilized anti-TCR- $\alpha/\beta$  mAb under conditions known to induce CD40L expression and maintain CD4 expression in normal animals. CD40L expression was determined by double staining of the activated LN T cells with CD4-FITC and with huCD40.Fc-biotin plus PE-conjugated streptavidin, followed by two-color flow cytometric analysis. Results indicated that 26.3% of activated CD4<sup>+</sup> LN T cells from littermate controls displayed binding of CD40.Fc relative to binding of a control protein (IL-4R.Fc) (Fig. 2, A and B). This is consistent with previous reports demonstrating that a subset of CD4<sup>+</sup> T cells express CD40L in response to anti-TCR stimulation (13, 15, 32). By contrast, the activated CD4<sup>+</sup> LN T cells from CD40LKO mice failed to bind detectable levels of CD40.Fc, indicating a total absence of functional CD40L expression on the cell surface (Fig. 2, C and D). No detectable ligand expression was observed on either control- or CD40LKO-derived CD4<sup>+</sup> T cells cultured in medium alone (data not shown).

Flow cytometric analysis was performed on cells from naive CD40LKO and naive control mice in order to compare leukocyte subpopulations. Relative to controls, CD40LKO-derived spleens and LNs contained similar percentages of B cells (as measured by surface IgD, surface IgM, and B220

wild-type allele. (E) Southern blot analysis of the mutated CD40L gene. Genomic DNA (15  $\mu$ g) was digested with PstI, electrophoresed through agarose, blotted to nitrocellulose, and hybridized to the probe indicated above. Positions of hybridizing bands (2.2 kb for the mutant allele;  $\sim$ 9.0 kb for the wild-type allele) are indicated.



**Figure 2.** CD40L expression is undetectable on activated CD4<sup>+</sup> LN T cells obtained from CD40LKO mice. LN T cells obtained from CD40LKO and control mice were activated for 18 h with immobilized anti-TCR mAb and double stained with either huIL-4R.Fc-biotin (A and C) or huCD40.Fc-biotin (B and D) and CD4-FITC (A–D) as described in Materials and Methods. The results are representative of the staining on three CD40LKO mice and two control mice.

expression) and T cell subsets (as measured by CD3, CD4, and CD8 expression) (Table 1). These tissues also contained normal ratios of CD4<sup>+</sup> and CD8<sup>+</sup> populations. Results from four additional experiments were consistent with these findings. Thus, there did not appear to be any gross phenotypic differences in lymphocyte subpopulations between CD40LKO and normal mice. However, there were differ-

ences in numbers of cells recovered from CD40LKO LNs when compared with control littermate LNs. The results in Table 2 show that total LN cellularity was approximately two- to threefold lower in CD40LKO mice.

**Humoral Immune Responsiveness.** Although most HIGM patients display elevated levels of circulating IgM compared with normal subjects, they are severely deficient in other serum isotypes. To determine whether CD40LKO mice exhibit the same phenotype, serum was collected from two groups of mice (control and CD40LKO) at 30, 44, 58, and 72 d of age, and isotype concentrations were determined by ELISA. Results in Fig. 3 demonstrate that IgM levels were essentially identical between the two groups at all ages. IgG3 levels were similar between the two groups although older mice displayed some differences ( $p < .038$  between CD40LKO and control mice on day 72). Differences in serum levels were more pronounced for the other isotypes, with considerably lower IgA ( $p < .004$ ), IgG1 ( $p < .026$ ), and IgG2b ( $p < .028$ ) in CD40LKO mice on day 72. In this and several other experiments, the CD40LKO mice consistently demonstrated a complete absence of IgE (detection limit of 0.02 ng/ml); by contrast IgE is always detectable in serum from control mice, although as demonstrated in this experiment the levels may vary considerably between individual mice.

The ability of B cells to secrete antibody and undergo class switching in response to certain antigens, particularly soluble protein antigens, requires T cell help that is at least in part mediated through CD40L–CD40 interactions (18, 33). To test the hypothesis that CD40LKO mice are incapable of responding to a typical “T-dependent” Ag, CD40LKO, and control mice received primary (day 0) and secondary (day 21) injections of TNP-KLH in alum, then were bled 5 d later to measure anti-TNP antibody levels by ELISA. Control mice produced significant levels of anti-TNP IgA, IgE, IgG1, IgG2a, IgG2b, IgG3 (Table 3), and IgM (Fig. 4); in contrast,

**Table 1.** Cell Surface Marker Analysis of Spleen and LN Cells Isolated From Control or CD40LKO Mice

	B cell subset			T cell subset			Mac subset
	IgD <sup>b+</sup>	IgM <sup>+</sup>	B220 <sup>+</sup>	CD3 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	Mac-1
<b>Spleen</b>							
Control no. 1	33	70	62	27	17	12	8
Control no. 2	55	63	55	36	24	12	10
CD40LKO no. 1	31	67	58	31	19	13	7
CD40LKO no. 2	40	74	65	22	14	8	10
<b>Lymph node</b>							
Control no. 1	15	34	35	67	43	24	4
Control no. 2	30	35	31	65	45	21	3
CD40LKO no. 1	15	35	33	64	46	18	3
CD40LKO no. 2	19	44	37	56	36	21	2

Numbers represent percent positive cells for the surface protein being examined. Gating parameters were dependent on autofluorescence and isotype control antibody binding.

**Table 2.** Lymph Node Cellularity in Control and CD40LKO Mice

		Total Cells × 10 <sup>-6</sup> *	$\bar{x}$ †
Expt. 1 (popliteal, inguinal, brachial)	Control no. 1	1.8	2.1
	Control no. 2	2.5	
	CD40LKO no. 1	0.9	0.9
	CD40LKO no. 2	1.0	
Expt. 2 (axillary, brachial, cervical, inguinal, periaortic)	Control no. 1	23.2	26.2
	Control no. 2	29.2	
	CD40LKO no. 1	7.0	7.3
	CD40LKO no. 2	7.5	
Expt. 3 (axillary, brachial, cervical, inguinal, periaortic)	Control no. 1	31.9	
	Control no. 2	24.4	24.8
	Control no. 3	18.0	
	CD40LKO no. 1	10.2	
	CD40LKO no. 2	4.5	7.8
	CD40LKO no. 3	8.8	

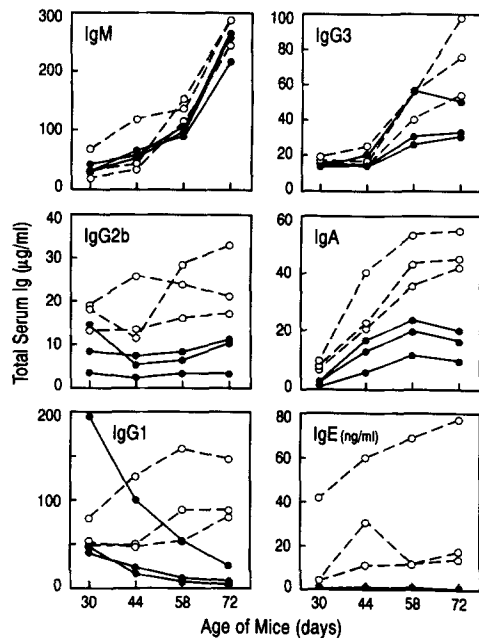
\* Numbers represent total cell number recovered from indicated lymph node sources (axillary, brachial, etc.) per individual mouse. Data from three separate cohorts of mice are presented.

†  $\bar{x}$ , mean total cell number per group.

CD40LKO mice failed to mount an anti-TNP secondary response of all isotypes except IgM. In the latter case, the CD40LKO mice developed a low IgM anti-TNP response in the first of two independent experiments (Fig. 4). In a second experiment, one of three CD40LKO mice exhibited

an IgM Ag-specific response that was comparable to that of one of the control mice. These results demonstrate that CD40L-deficient mice are incapable of undergoing Ig class switching but can secrete a variable amount of IgM in response to a TD Ag.

Certain antigens such as bacterial polysaccharides are capable of eliciting humoral immune responses in the apparent absence of T cell help (34–36). The ability of CD40LKO mice to produce specific antibody in response to a T independent Ag was assessed as follows. Mice were immunized with DNP-Ficoll, a TI-2 Ag, and bled 10 d later for determination of anti-TNP antibody levels. Serum from both CD40LKO and control mice exhibited strong IgM and IgG3 anti-TNP titers after DNP-Ficoll immunization (Fig. 5). Both sets of mice also produced detectable titers of IgA, IgG1, and IgG2b anti-TNP, a result which was reproduced in a second experiment (data not shown). There were notable differences in the titration curves between control and CD40LKO sera. For example, in the case of IgG2b anti-TNP the serum samples from the



**Figure 3.** Total Ig isotypes in nonimmune serum. Control (open symbols) and CD40LKO mice (solid symbols) were bled successively at 30, 44, 58, and 72 d of age. An isotype-specific sandwich ELISA technique was used to assay serum samples (1:10 to 1:600,000 dilutions) as described in Materials and Methods. Values are means ( $\pm$  SEM) from each group and are presented as  $\mu$ g/ml (ng/ml for IgE).

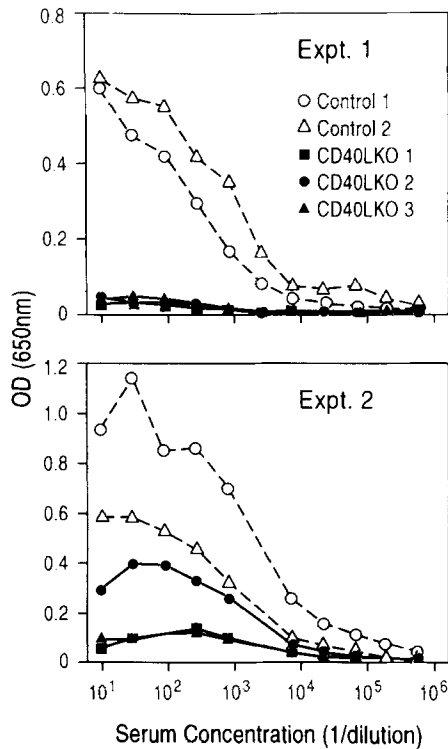
**Table 3. CD40LKO Mice Fail To Generate Secondary TD Ag-specific Antibody Responses**

Isotype	Serum anti-TNP titers (U/ml)*		
	CD40LKO (n = 3)	Control no. 1	Control no. 2
IgA	<10	9,485	457
IgE	<10	6,978	2,003
IgG1	<10	626,000	140,000
IgG2a	<10	64,275	2,661
IgG2b	<10	149,000	34,066
IgG3	<10	316	5,858

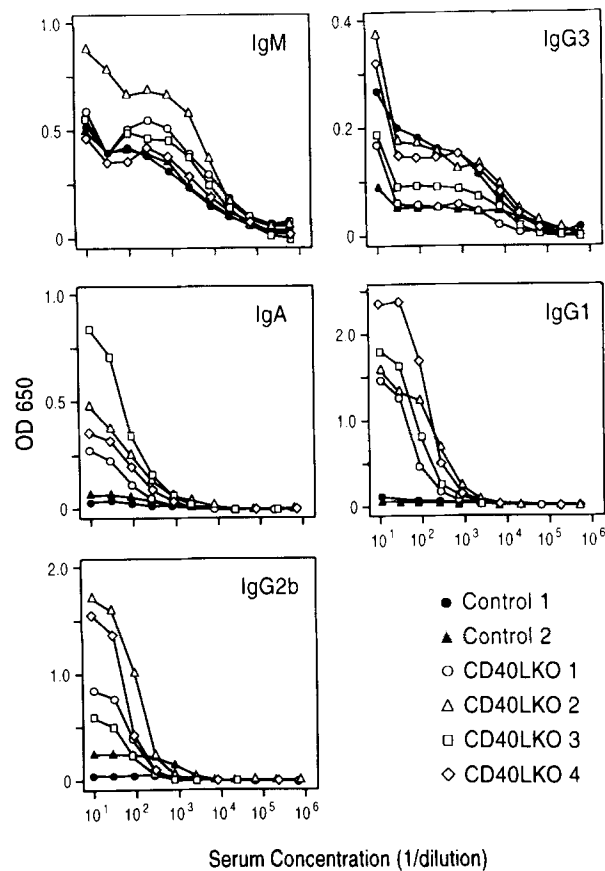
\* Control and CD40LKO mice were primed and challenged with TNP-KLH. Serum anti-TNP isotype levels were determined by ELISA and data were analyzed as described in Materials and Methods. Results are presented as U/ml based upon the dilution giving a half-maximal OD value relative to an arbitrarily selected maximum value.

four CD40LKO mice exhibited considerably higher maximum OD values relative to controls (Fig. 5). In contrast, control and CD40LKO sera had essentially identical endpoint titers. This difference is likely due to the nature of the "capture" ELISA assay, in which total Ig isotype is captured before the addition of Ag (TNP-BGG). In the experiment summarized in Fig. 5, total serum IgG2b levels were considerably higher in control animals (mean = 213  $\mu\text{g/ml}$ ) than in CD40LKO

animals (mean = 8  $\mu\text{g/ml}$ ). Therefore, if anti-TNP levels were equivalent in both CD40LKO and control sera, then the percent of total IgG2b that was TNP-specific would be  $\sim 25$ -fold lower in the control mice than in CD40LKO mice.



**Figure 4.** CD40LKO mice generate weak secondary IgM anti-TNP-KLH antibody responses. Mice (two control and three CD40LKO each in two independent experiments) were immunized with TNP-KLH and assayed for IgM anti-TNP responses as described in Table 3. Results are presented as OD units from ELISA assays.



**Figure 5.** CD40LKO mice secrete multiple isotypes of Ag-specific antibody in response to a TI-2 Ag. Control and CD40LKO mice were injected intraperitoneally with 10  $\mu\text{g}$  DNP-Ficoll. Mice were bled 10 d later and serum anti-TNP isotype responses were determined by ELISA as described in Table 3. Each line represents an ELISA titration curve from an individual animal.

**Table 4.** CD40LKO B Cell Proliferative Responses\*

Stimulus	+ IL-4/IL-5	<sup>3</sup> H]TdR incorporation	
		CD40LKO	Control
<i>cpm</i>			
None	-	76 (22)	72 (15)
	+	1,494 (131)	2,659 (62)
LPS (1 µg/ml)	-	20,508 (608)	25,024 (629)
	+	26,598 (1,110)	28,430 (2,922)
Anti-IgM (10 µg/ml)	-	799 (59)	1,775 (93)
	+	7,356 (969)	10,790 (1,893)
CD40L (3 µg/ml)	-	2,284 (162)	3,337 (44)
	+	19,674 (1,454)	21,112 (1,778)

\* Splenic B cells from CD40LKO and control mice were cultured for 3 d with various stimuli and proliferation was measured as incorporation of tritiated thymidine as described in Materials and Methods. Data are presented as mean cpm ( $\pm$  SEM) for triplicate samples.

Thus, at higher serum concentrations where saturation of the anti-IgG2b capture step is occurring, the detectable levels of anti-TNP IgG2b would be underestimated in the control mice. This interpretation notwithstanding, the results demonstrate that CD40LKO mice are capable of mounting a humoral immune response to a TI Ag and can switch to isotypes other than IgM.

**CD40LKO-derived B Cell Responses In Vitro.** Splenic B cells were purified from CD40LKO mice in order to assess their ability to respond to stimulators of B cell growth and differentiation in vitro. As shown in Table 4, B cells from CD40LKO and control mice exhibited comparable proliferative responses to LPS, anti-IgM, and recombinant soluble CD40L in the presence and absence of cytokines (IL-4 plus IL-5). B cells from CD40LKO and control mice were also tested for their ability to secrete Ig in response to cytokines plus LPS or CD40L. CD40LKO-derived B cells are capable of responding

to either stimulus, although the relative levels of IgM, IgE, and IgG1 were somewhat lower than was seen in control B cells (Table 5). The results indicate that the CD40LKO mice retained a capacity for Ig class switching despite the absence of CD40L expression in vivo.

**Histological Findings in the LNs and Spleens of Immunized CD40LKO Mice.** Inguinal LNs and spleens were obtained from CD40LKO mice and littermate controls that had been immunized and challenged with the Ag TNP-KLH. Hematoxylin and eosin staining revealed the presence of primary follicles in the LNs of both immunized CD40LKO mice and control mice. Prominent germinal center (secondary follicle) formation, however, was only observed in the LN and spleen of littermate control mice (Fig. 6, A and B). No germinal center formation could be observed in either the LN or the spleen of immunized CD40LKO mice (Fig. 6, C-E). Other histological findings in the LN and spleen in the CD40LKO mice were unremarkable when compared with littermate controls.

## Discussion

CD40L is an inducible type II membrane glycoprotein found on the surface of T cells after antigenic stimulation. Although CD40L has diverse activities including the induction of B and T cell proliferation, cytokine production from T cells, monocytes, and plasma cells, and macrophage tumoricidal activity (15, 19-22, 37), these effects are also induced by several other cytokines. CD40L has a unique role in the initiation of B cell affinity maturation and isotype switching that occurs in the germinal centers of secondary lymphoid tissue after antigenic challenge. Evidence for the essential requirement of CD40L in this process came from studies of HIGM patients who lack functional CD40L (38). These individuals are unable to produce high affinity Ag-specific Ig of isotypes other than IgM and lack germinal centers.

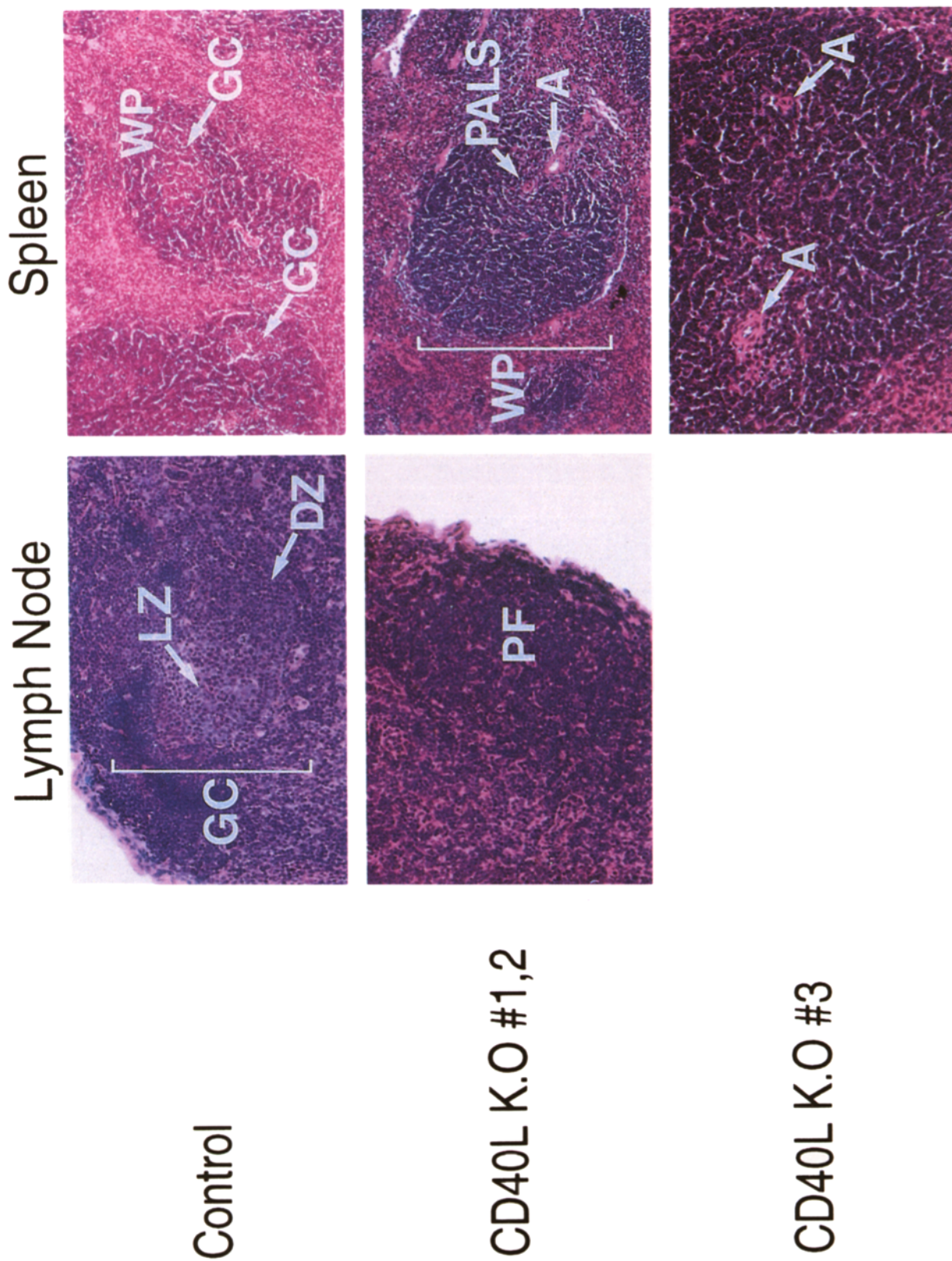
In this report, we describe the generation of CD40LKO mice in which the CD40L gene has been disrupted, resulting in a failure to express a functional CD40L protein on the

**Table 5.** CD40LKO B Cell Immunoglobulin Secretion In Vitro\*

Costimulus	IgM		IgG1		IgE	
	CD40LKO	Control	CD40LKO	Control	CD40LKO	Control
None	2	1	ND	ND	ND	ND
LPS	28,145	46,416	361	651	288	509
	(585)	(6)	(15)	(73)	(27)	(132)
CD40L	12,906	24,140	1,562	1,702	3,913	6,816
	(1,104)	(1,671)	(329)	(103)	(265)	(1,332)

\* Splenic B cells from CD40LKO and control mice were cultured for 6 d with either purified recombinant CD40L (3 µg/ml), LPS (10 µg/ml), or no costimulus. All cultures contained IL-4 and IL-5, (10 ng/ml each). Culture supernatants were assayed for Ig isotype levels by ELISA as described in Materials and Methods. Data are presented as mean ng/ml ( $\pm$  SEM) for triplicate samples. ND, below detectable limits.





**Figure 6.** Germinal center formation is not observed in CD40LKO mice after Ag challenge. Mice were immunized with TNP-KLH as described in Fig. 4. LNs and spleen were stained with hematoxylin and eosin and analyzed histologically for the presence of germinal centers. The following abbreviations are used: GC, secondary follicle with germinal center; LZ, light zone; DZ, dark zone; A, arteriole surrounded by lymphatic sheath; WP, white pulp; PF, primary follicle, PALS, periaarteriolar lymphocyte sheath. The original magnification is either 20× or 40×.

cell surface. When immunized with a TD Ag, these mice resemble their human counterparts with HIGM syndrome; they are unable to form germinal centers and produce only Ag-specific IgM. Despite such defects in isotype switching, B cell development appears unaffected in these mice, which have relatively normal percentages of B cells in the circulation and lymphoid tissues. Furthermore, B cells isolated from CD40LKO mice are able to proliferate and secrete a profile of Ig isotypes comparable to control mice when cultured with recombinant CD40L or LPS and cytokines, indicating that the defect in Ig production seen in these mice is restricted to the TD help provided during B cell differentiation. Despite CD40L having stimulatory activity on T cells (20, 22), these populations in the CD40LKO mice appear normal with respect to proliferative responses to concanavalin A and phytohemagglutinin (data not shown).

Serum levels of different Ig isotypes in the unimmunized CD40LKO mice were compared with those of normal control animals. As CD40LKO mice matured, their sera contained significantly lower amounts of IgG1, IgG2b, and IgA relative to controls, and no detectable IgE. IgG3 levels were somewhat reduced at 72 d of age, but the levels of IgM present were essentially normal. This profile of murine serum Ig isotypes is qualitatively similar to that reported for HIGM patients, although the effect in the latter is quantitatively more dramatic. It will be important to determine whether the murine serum isotype differences become more pronounced, and thus more reflective of the HIGM serum profile, with further aging. It is noteworthy that an age-dependent decrease in serum IgG1 was observed in CD40LKO mice. This presumably reflects the presence of maternal IgG1 still in the circulation of young mice (39–41).

The defects in response to TD Ag in CD40LKO mice contrast with their response to TI Ag. TI-2 antigens such as bacterial polysaccharides are able to induce a strong humoral response *in vivo* in the absence of T cells (34–36). Immunization of mice with TI-2 antigens results in significant levels of Ag-specific IgM and IgG3 in the serum (34, 42, 43). It is clear, however, the Ag alone is insufficient to induce Ig secretion; soluble cytokines are required which regulate the profile of isotypes produced. When immunized with DNP-Ficoll, both CD40LKO and control littermates produced strong IgM and IgG3 anti-TNP titers, typical of responses

to this antigen. It is interesting to note that both sets of immunized mice produced detectable amounts of IgG1, IgG2b, and IgA anti-TNP antibody but no TNP-specific IgE. The ability of the CD40LKO mice to generate anti-TNP Ig of isotypes other than IgM confirms Ig isotype switching can be induced in response to Ag in the absence of CD40L. These findings concur with those of previous studies with normal mice where anti-IgD conjugated to high molecular weight dextran has been used to mimic the repeating epitopes common in bacterial polysaccharide antigens (42, 43). In the presence of IL-5, anti-IgD-dextran can stimulate resting B cells to secrete high levels of IgM and IgG1, or IgG3 and IgG2a in the presence of IFN- $\gamma$  in a T cell-independent manner (44, 45). In contrast to B cells stimulated with LPS, the combination of anti-IgD-dextran and IL-5 does not induce IgE secretion in the presence of IL-4 (45, 46).

It has recently been demonstrated that B-1 (CD5<sup>+</sup>) B cells, when activated with LPS, can undergo isotype switching in the presence of IL-4 and IL-5 (47). Although the origin of B-1 B cells remains controversial, it has been suggested that cells of this phenotype may arise following interaction with TI-2 Ag in the absence of any contact with helper T cells (48, 49). It is possible therefore, that the humoral response to TI-2 Ag observed in both CD40LKO and control mice is mediated by B-1 B cells. However, in neither CD40LKO nor control mice immunized with TNP-Ficoll was an increase in the proportion of B cells expressing CD5 observed, compared with that of untreated animals (data not shown). It remains to be determined whether Ig secretion is elevated from B-1 B cells following immunization with TI-2 Ag.

In summary, mice lacking CD40L are unable to undergo Ig isotype switching in response to a T-dependent Ag and fail to develop germinal centers upon antigenic stimulation. In contrast, the response to T-independent Ag appears to be unimpaired, with the production of Ag-specific IgG evidence that isotype switching can occur in the absence of CD40L. CD40LKO mice constitute a model which will be of value in determining the roles that CD40L, related molecules and soluble cytokines play at critical stages of the immune response, and will help to define the function of molecules that share certain biological activities of CD40L.

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