

# Memory B Cell Development but Not Germinal Center Formation Is Impaired by In Vivo Blockade of CD40-CD40 ligand Interaction

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## Summary

To study the role of the CD40-CD40 ligand interaction in the development of memory B cells and its level of action during primary antibody responses in vivo, mice were injected with a soluble CD40 fusion protein (sCD40- $\gamma$ 1), so as to block the interaction. The effects of the treatment on the primary antibody response were reminiscent of hyper-immunoglobulin M (IgM) syndrome (HIMG1): antigen-specific IgG responses were grossly inhibited whereas the IgM response was augmented severalfold. The latter observation suggests that there is a T-dependent, CD40 ligand-independent pathway of B cell activation that leads to IgM responses and that a significant component of the IgM in HIMG1 patients is derived from T-dependent responses. The secondary response was not readily blocked by sCD40- $\gamma$ 1 treatment, indicating a relative independence of CD40 ligation of antigen-experienced B cells. The most striking finding from these studies is that the development of memory B cell populations (measured by adoptive transfer) is grossly impaired by administration of sCD40- $\gamma$ 1 during the early induction phase of the response. It is surprising that although the generation memory is diminished, there is no quantitative difference in the development of germinal centers. Whereas entry of B cells into the memory cell pathway is dependent on CD40 ligation, the clonal expansion of the potential memory precursors in germinal centers seems not to require a CD40 signal.

The encounter of resting B cells with thymus-dependent antigens neither causes them to enter cell cycle nor to become responsive to lymphokines. For the induction of responses to these very common antigens (virtually all protein antigens), B cells require additional stimuli to those received via sIg if they are to accept the differentiation signals delivered by T cell-derived lymphokines. In the mid-1980s it became clear that the source of these "accessory" or "costimulatory" signals was membrane-bound molecules on activated T cells. This was demonstrated first by using fixed, activated T cells in coculture with B cells (1, 2) and subsequently, purified cell membranes from such cells (3-5). More recently, several laboratories have demonstrated that CD40 is among the most important B cell acceptor molecules acting during this interaction. Genetically engineered, soluble forms of CD40 largely block the B cell proliferation induced by fixed, activated T cells (6-8).

CD40 is expressed ubiquitously throughout the B cell lineage (9), as well as on interdigitating dendritic cells and on some epithelial cells (10). It is grouped, on the basis of sequence and structural homology, in the nerve growth factor family of receptors, which also includes TNF- $\alpha$  receptor (11), CD27 (12), CD30 (13), and the Fas antigen (14). Antibodies to CD40 have been shown to be directly mitogenic for B

cells, and when immobilized on the surface of Fc receptor-bearing fibroblasts, will support the long-term growth of nontransformed B cell lines (15). The growth-mediating effects of anti-CD40 are enhanced in the presence of other cofactors (e.g., IL-4 and anti-Ig; 16-18). In particular, the addition of lymphokines to these cultures induces differentiation, isotype switching, and antibody secretion (19-21). The molecular cloning of the physiological ligand for CD40 (22-24) showed it to be a type II membrane molecule, with homology to TNF ( $\alpha$  and  $\beta$ ), that is expressed after activation of almost all CD4<sup>+</sup> T cells and a minority population of CD8<sup>+</sup> T cells (25).

Confirmation that interaction between CD40 and its ligand was pivotal in T-dependent B cell antibody responses came last year with a number of groups demonstrating mutations in the CD40-ligand (CD40-L)<sup>1</sup> gene as the molecular basis

<sup>1</sup> Abbreviations used in this paper: CD40-L, CD40-ligand; GC, germinal center; HIMG1, X-linked immunodeficiency with hyper-IgM; PNA, peanut agglutinin; TD, thymus dependent; sCD40- $\gamma$ 1, soluble mouse CD40-human Fc $\gamma$ 1 fusion protein.

ular basis of the lesion in the X-linked form of a rare immunodeficiency condition known as Hyper-IgM Syndrome (HIMG1) (26–29). Patients with this disease, among other problems, exhibit almost no serum Ig of the “switched” classes (i.e., IgG, A, or E), but have elevated levels of IgM and sometimes IgD. It is a matter of contention whether the serum IgM in HIMG1 patients is made up of natural antibodies (derived from CD5 B cells [30]) or results from responses to thymus-independent antigens. A component may be thymus dependent (TD), as patients are reported to respond after primary immunization with TD antigens, albeit only in the IgM class (21, 31). It would seem, therefore, that T-dependent, antigen-specific IgM responses can occur in the absence of CD40 ligation. This conclusion is challenged by data reported by Korthäuer et al. (26): B cells from HIMG1 patients fail to produce IgM after polyclonal stimulation with PWM unless antibodies to CD40 are added to the culture. This in turn contradicts a number of previous studies that show no defect in PWM-stimulated IgM production by HIMG1 B cells (32–34), even though it is known to be T dependent in nature (35). Whichever of these results is correct, the current wisdom is that signals received through CD40 are crucial for the development of T-dependent antibody responses.

In addition to its roles in B cell activation and isotype switching discussed above, antibodies to CD40 deliver very potent rescue/survival signals to germinal center (GC) B cells that are susceptible to apoptosis (36). As part of the process of B cell memory generation, B cells proliferate and somatically mutate in GC (37, 38). During this period they are exquisitely sensitive to apoptosis, in all likelihood because of a loss of bcl-2 expression (39). Rescue with antigen, although an important first step in the selection of high affinity mutants, is a much less potent survival signal than ligation of CD40 (36). The cellular product of the CD40 rescue, *in vitro*, is a small, nondividing lymphocyte. This has led to the speculation that the CD40 signal, presumably in the absence of lymphokines, drives B cells into the memory pathway. The information available from HIMG1 patients is not conclusive concerning the generation of memory in the absence of CD40 ligation. Booster injections of vaccines continue to elicit IgM responses, with only a few cases yielding IgG (31), but there has been little investigation of whether this represents a memory response.

A lack of direct evidence concerning the role of CD40 ligation in the development of B cell memory and a wish to learn about the exact level of action of CD40-L *in vivo*, given the many diverse functions assigned to it, led us to try to block the CD40–CD40 ligand interaction by injecting mice with a soluble form of mouse CD40 (a chimeric molecule with human Fc $\gamma$ 1) during immune responses. We felt that such an approach would extend the knowledge derived from HIMG1, in that we could confine the blockade of CD40-L to defined periods of the immune response and so avoid any gross defects arising out of long-term inactivation of a multifunctional receptor. The experiments highlight an important role for CD40 ligation in the differentiation to memory B cells but suggest this is separate from the initiation of clonal expansion of memory precursors in GC.

## Materials and Methods

**Construction of Soluble Mouse CD40-Human IgG1 Chimeric Protein.** The primers used to amplify the extracellular portions of the mouse CD40 gene (40) were 5'(GACTGAGCTCACCATGGTGTCCTTTCGCTCGGCTGTG) and 3'(TATACTTACCCATCCGGGACTTTAAACCACAG). cDNA was prepared from mouse splenic B cells (reagents purchased from Promega Corp., Madison, WI) and used as a template for PCR amplification. Each primer contained restriction sites that enabled subcloning into the human IgG1 expression vector (7), together with a 3' splice donor site in the 3' primer to facilitate correct splicing to the human  $\gamma$ 1 exons. The resulting plasmid was used to transform K803 bacteria.

**Transfection and Purification.** J58L plasmacytoma cells were transfected with the mCD40-hu $\gamma$ 1 plasmid by protoplast fusion. Cells were plated in flat-bottomed 96-well plates at  $5 \times 10^4$ /ml in IMDM plus 10% FCS plus 10  $\mu$ g/ml mycophenolic acid (Calbiochem-Novabiochem Corp., La Jolla, CA) plus 250  $\mu$ g/ml xanthine (Sigma Chemical Co., St. Louis, MO). Clones arising around 2 wk later, after several renewals of selective media, were screened for the production of human IgG1 in the supernatants. Soluble mouse CD40-hu $\gamma$ 1 was purified from large volumes of culture supernatants, after ammonium sulphate precipitation, by binding to protein A columns (Pharmacia, Uppsala, Sweden) and elution at pH 3.5.

**T-dependent B Cell Proliferation Assay.** The I-A<sup>d</sup>-restricted sperm whale myoglobin-specific Th1 clone, 11.3.7 (kindly provided by Dr. Alexandra Livingstone, Basel Institute for Immunology, Basel, Switzerland) was taken in a “resting” state (2 wk after its last antigenic stimulation) and activated for 18 h with 10 ng/ml PMA and 1  $\mu$ g/ml ionomycin. Cells were washed and then fixed in 0.8% paraformaldehyde as previously described (1). These fixed, activated T cells were placed in flat-bottomed 96-well plates at  $10^5$  per well together with  $10^5$  high density DBA/2 splenic B cells taken from the 1.085–1.092-density interface of a Percoll gradient. These B cells had been extensively depleted of T cells using a cocktail of IgM antibodies to CD4, CD8, and Thy1 plus rabbit complement followed by IgG antibodies to the same antigens coated onto Dynabeads (Dynal, Oslo). In some wells, anti-Ig antibodies (monoclonal anti- $\kappa$ ; 187.1) or supernatant containing IL-4 (41) was added at 10  $\mu$ g/ml and 1%, respectively. Cultures in IMDM plus 10% FCS plus 2 mM L-glutamine plus 100 U/ml penicillin plus 100  $\mu$ g/ml plus 50  $\mu$ M 2-ME, were continued for 66 h with [<sup>3</sup>H]thymidine (2  $\mu$ Ci/well) added for the final 18 h. Soluble mCD40-hu $\gamma$ 1 was added to cultures at concentrations varying from 0.1 to 10  $\mu$ g/ml; maximal inhibition was observed at 2.5  $\mu$ g/ml.

**In Vivo Administration of Soluble CD40-hu $\gamma$ 1.** Mice were immunized intraperitoneally with 50  $\mu$ g of alum-precipitated antigen (except C5, see below, of which 10  $\mu$ g was injected). Groups of five or six immunized mice were injected intravenously either with sCD40- $\gamma$ 1 or with purified human IgG1 myeloma protein (The Binding Site, Birmingham, UK) at 100  $\mu$ g/mouse/d. The injection of these substances was continued for 5 d or 10 d, or in some cases the onset delayed until day 4 after immunization and continued for an additional 4 d. The antigens used in these studies were dinitrophenylated OVA, DNP-KLH (Calbiochem-Novabiochem Corp.), native KLH, and the mouse complement component C5 (a gift from Dr. Brigitta Stockinger, National Institute for Medical Research, Mill Hill, London, UK). All antigens were coupled to DNP as previously described (42). In one experiment, mice were injected subcutaneously first with alum-precipitated antigen and then with four daily injections of sCD40- $\gamma$ 1 on the upper surface of the front legs. The mice were bled at various times after immunization. Mice used for these experiments were BALB/c and

DBA/2 supplied from the Specific Pathogen Free Unit at NIMR and maintained under normal laboratory conditions in the Royal Postgraduate Medical School animal facility.

**Adoptive Transfers to Measure Memory Responses.** BALB/c mice (IgH<sup>b</sup> allotype) treated with soluble mCD40-hu $\gamma$ 1 or controls treated with huIgG1 during primary responses to DNP-OVA were left for 10 wk. At this time, spleen and peripheral lymph nodes were taken from these mice, suspensions made, and  $2 \times 10^6$  or  $5 \times 10^6$  cells (see legend to Fig. 6) were injected intravenously into 5 Gy-irradiated C.B20 mice (IgH<sup>b</sup> allotype), together with  $5 \times 10^6$  spleen cells from KLH-primed C.B20 mice. The mice were immunized with 10  $\mu$ g i.v. of soluble DNP-KLH. Cell suspensions from mice treated similarly were pooled before reconstitution. Mice were bled at 7 and 14 d after this and the sera assayed for donor anti-DNP antibodies.

**IL3 Limiting Dilution Assays.** The frequency of OVA-specific T cells was measured using an assay almost exactly as described by Ronchese and Hausmann (43). The only differences were that we used the PBC3 IL-3-dependent cell line (44), adding 50 cells to the wells after the plates were irradiated. After 24 h, a rescue dose of IL-3 was added (1% of IL-3 containing supernatant; 41) and culture continued for 4–5-d more, with [<sup>3</sup>H]thymidine (Amersham International, Amersham, Bucks, UK) added for the final 16 h. Frequencies were calculated from the regression curve as described (43), and results were presented as frequency per total splenic T cell population.

**Antibodies, Flow Cytometry, and Immunohistology.** FITC-labeled anti-CD25 (3C7; reacting with the p55, low affinity IL-2 receptor) was purchased from PharMingen (San Diego, CA). GK1.5 (anti-CD4) was obtained from the American Type Culture Collection and biotinylated using biotin-N-hydroxysuccinimide ester (Calbiochem-Novabiochem Corp.); this was detected using streptavidin-PE (Southern Biotechnology Associates, Birmingham, AL). CD40-L expression was detected using biotinylated sCD40- $\gamma$ 1 (a blocking step with 2% human serum was included and CD40-hu $\gamma$ 1-biotin was diluted in PBS containing 2% human serum to avoid the possibility of Fc binding). Otherwise, cells were stained in standard fashion and analyzed on a flow cytometer (Epics XL; Coulter Corp., Hialeah, FL). For assessment of GC formation, 5- $\mu$ m-thick frozen sections of spleen were acetone fixed and stained with biotin-labeled peanut agglutinin (PNA; Vector Labs, Peterborough, UK) and horseradish peroxidase-labeled streptavidin (Southern Biotechnology Associates).

**Determination of Antibody Titers.** Microtiter ELISA plates (Dynatech Laboratories, Chantilly, VA) were coated with the test antigen (DNP-bovine serum antigen, KLH, or C5) at 10  $\mu$ g/ml and 50  $\mu$ l/well in carbonate/bicarbonate buffer, pH 9.5. Assays were carried out and antibody titers worked out as previously described (42). Briefly, this involved doubling dilutions of serum, starting at 1 in 20. Curves of absorbance (at 405 nm) against serum dilution were plotted. The antibody titer was calculated as the reciprocal of the dilution that gave an absorbance of 30% of the maximum absorbance reading for that particular assay. The maximum was given by a standard hyperimmune serum. Secondary antiisotype antibodies used in these assays were the following: alkaline phosphatase conjugates of goat anti-mouse IgG1, goat anti-mouse IgG2a, goat anti-mouse IgM, and streptavidin (all purchased from Southern Biotechnology Associates). Anti-IgH<sup>b</sup> allotype antibodies RS3.1 (recognizes Igh6a on IgM; 45) and Ig(4a)10.9 (otherwise known as 20.9.10, recognizes Igh4a on IgG1; 46) were kind gifts from Drs. R. Palacios (Basel Institute for Immunology, Basel, Switzerland) and L. A. Herzenberg (Stanford University Medical School, Stanford CA), respectively. These were biotinylated for use

and detected using streptavidin-alkaline phosphatase. Plates were read on a Titertek Multiscan MkII ELISA plate reader (ICN Biomedicals, Costa Mesa, CA) at 405 nm.

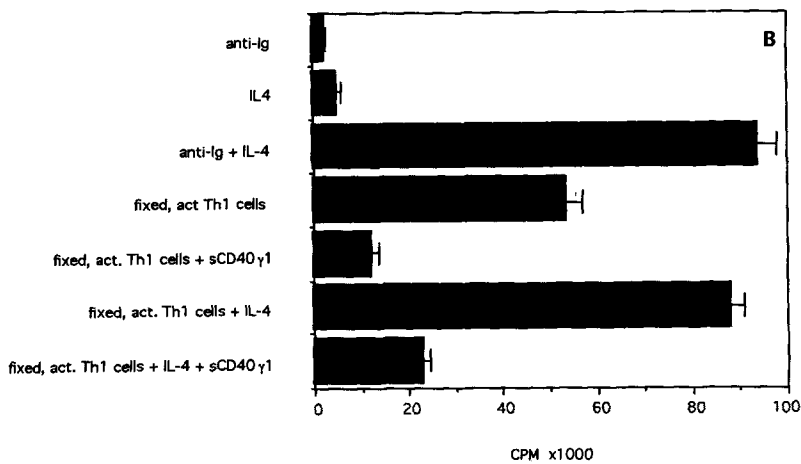
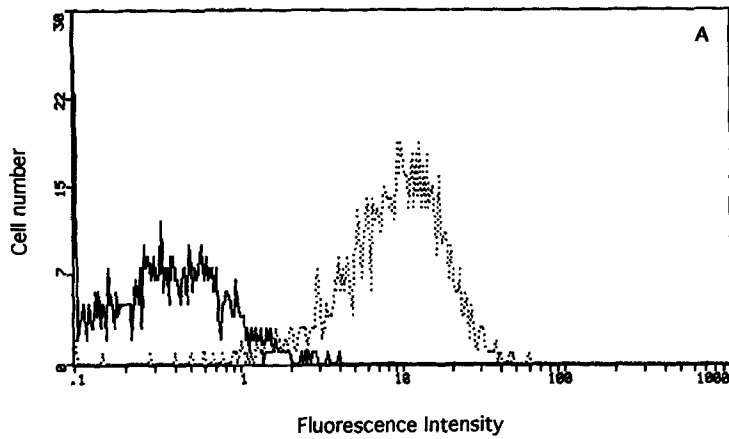
The half-life of huIgG1 or sCD40- $\gamma$ 1 in the serum was measured using the following ELISA: sheep anti-human IgG1 was coated onto wells at 10  $\mu$ g/ml. After blocking and incubation of serial dilutions of test sera, sheep anti-human IgG1 conjugated to alkaline phosphatase (The Binding Site) was added. The amount of human IgG1 in the sera was calculated from a standard curve generated from an ELISA using a titration of a human IgG1 myeloma protein (The Binding Site). Mouse antibody responses to the human Fc $\gamma$ 1 portion of the CD40-fusion protein were assayed by coating human IgG1 myeloma protein onto wells at 10  $\mu$ g/ml, incubating the test sera, and detecting bound mouse Ig with a goat anti-mouse Ig alkaline phosphatase conjugate absorbed against human Ig (Southern Biotechnology Associates).

## Results

**Soluble CD40- $\gamma$ 1 Inhibits T-dependent B Cell Proliferation In Vitro.** To ascertain whether the sCD40- $\gamma$ 1 would block the CD40-CD40 ligand interaction in vivo it was necessary to assess the capacity of the chimeric protein to inhibit the interaction in vitro. To this end it was introduced into a T cell-dependent B cell proliferation assay. The sperm whale myoglobin-specific Th1 clone 11.3.7 was stimulated overnight with PMA plus ionomycin. As shown in Fig. 1 A this induced the expression of the CD40-L. The cells were then fixed with paraformaldehyde and washed. Coculture of these fixed activated T cells with high density, resting B cells induces the B cells to proliferate (Fig. 1 B), a response that is enhanced by the addition of IL-4 or anti-Ig (data not shown). Addition of sCD40- $\gamma$ 1 to the cultures, at a concentration of 2.5  $\mu$ g/ml, inhibited the proliferation by  $\sim$ 75%. This is in line with previous observations (6–8).

**In Vivo Administration of Soluble CD40- $\gamma$ 1 during Primary Immune Responses.** DBA/2 mice were immunized with 50  $\mu$ g i.p. of alum-precipitated DNP-OVA and split into three groups. The first was injected intravenously on the day of immunization (day 1) and then every day until day 5 with 100  $\mu$ g of sCD40- $\gamma$ 1; the second received 100  $\mu$ g of purified human IgG1 with the same regimen (i.e., days 1–5); and the third received 100  $\mu$ g sCD40- $\gamma$ 1 starting on day 4 and continuing daily until day 8 after immunization. The rationale for these regimes was as follows: treatment from days 1–5 might be expected to influence the initiation of the antibody response whereas treatment from days 4–8 might affect the development of memory B cell populations in GC that first appear in the spleen at around day 7. The human IgG1 was used as a control for the potential effects of Fc receptor binding. We chose not to use a fusion protein that bound to another molecule on the T cell surface as this could introduce unknown effects into the control group.

Fig. 2, A and B shows the serum antibody response of these three groups of mice to DNP-OVA in the IgG1 and IgM classes. The IgG1 response, at day 7, was inhibited by 80% in the group given sCD40- $\gamma$ 1 from days 1–5 when compared with the group treated with huIgG1 (percent inhibition calculated from the means of the two groups). At day



**Figure 1.** Soluble CD40- $\gamma$ 1 stains an activated T cell clone and blocks T-dependent B cell activation in vitro. (A) FACS<sup>®</sup> plot of the binding of sCD40- $\gamma$ 1-biotin to the Th1 clone, 11.3.7, before (solid line) and 24 h after (dotted line) activation with PMA plus ionomycin. (B) Inhibition by sCD40- $\gamma$ 1 of B cell proliferation stimulated by fixed, activated Th1 clone, 11.3.7, either alone or together with IL-4. Although not shown, note that addition of huIgG1 to the cultures had no inhibitory effect on B cell proliferation. The mean  $\pm$  SD of triplicate wells are shown. The experiment is representative of three such experiments.

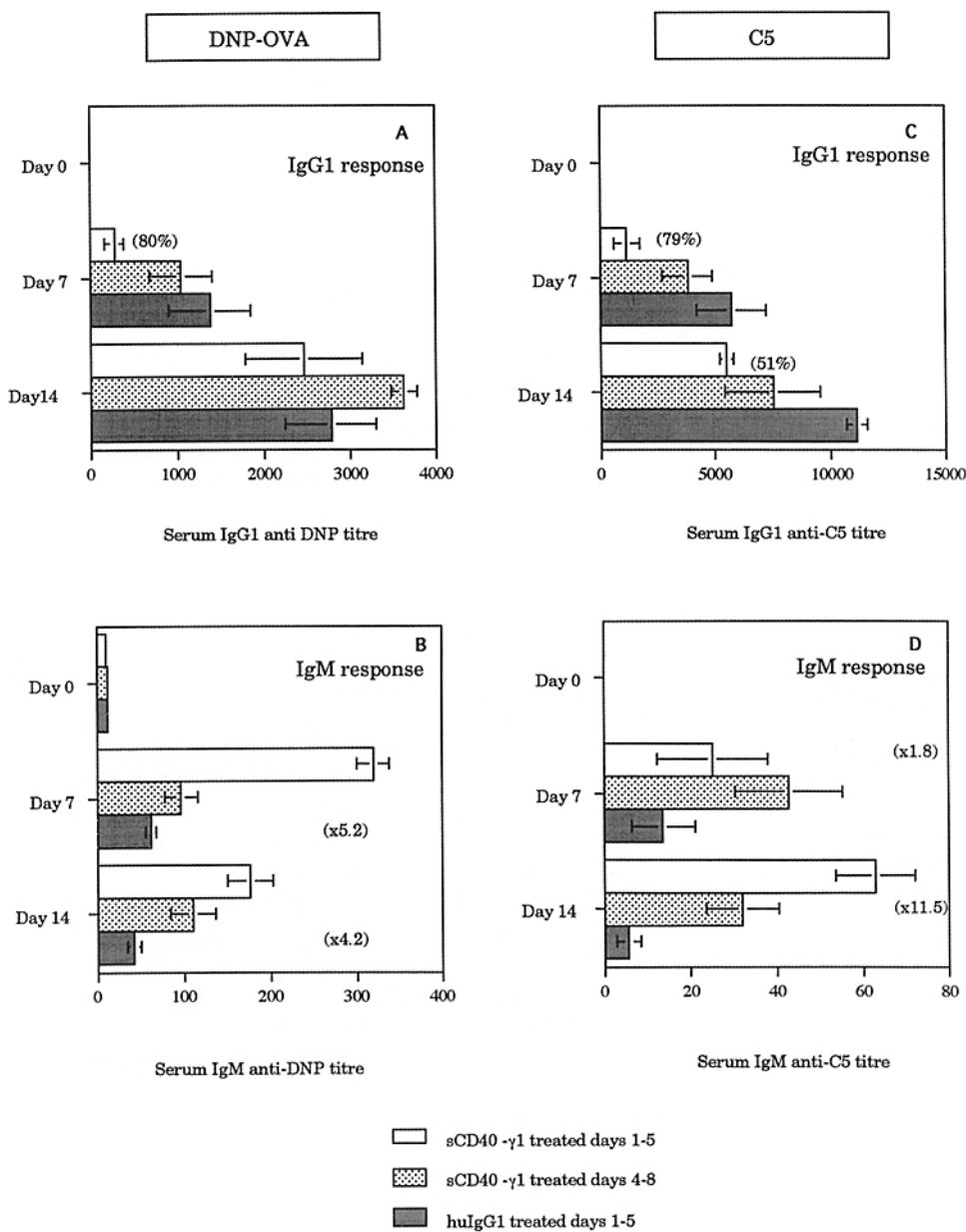
14 after immunization, these two groups show roughly equivalent responses. Delaying the onset of sCD40- $\gamma$ 1 treatment had minimal effect on the anti-DNP response at day 7 or 14 when compared with the control group. The IgM response in contrast to the IgG1 response was augmented by over fivefold in the mice treated with sCD40- $\gamma$ 1, in comparison with the huIgG1-treated animals (5.2-fold at day 7 and 4.2-fold at day 14). Again, delaying treatment until day 4 had only a marginal effect on the IgM response.

It should be noted that the IgG1 isotype dominates the response of normal DBA/2 or BALB/c mice to DNP-OVA, as it is at least 10-fold higher than the IgM or the IgG2a response. However, the sCD40- $\gamma$ 1-induced inhibition is not restricted to the IgG1 subclass (Th2-type response) as we found similar reductions in the IgG2a subclass (data not shown for this experiment, but see Fig. 3 B). The response to the complement component, C5, was investigated because in normal DBA/2 mice there is a very strong antibody response that is restricted to the IgG1 subclass, with virtually no IgM produced (DBA/2 mice are genetically deficient in C5, and therefore respond to it as a foreign antigen; 47). The responses to C5 in sCD40- $\gamma$ 1-treated mice are illustrated in Fig. 2,

C and D. The IgG1 response of mice treated from days 1-5 were inhibited by 79% at day 7 and by 51% at day 14, when compared with huIgG1-treated mice. Whereas the control group made a very poor IgM response to C5 (titer of 1 in 13), the sCD40- $\gamma$ 1-treated mice responded twofold better at day 7 and 11-fold better at day 14.

The effects of sCD40- $\gamma$ 1 injections were fairly short-lived; to see if "suppression" could be prolonged we injected mice for the first 10 d of the response to DNP-OVA. Fig. 3, A-C shows that the inhibition of the IgG1 or IgG2a responses were maintained at least until day 14 in this experiment (IgG1, 78% inhibition at day 7 and 61% at day 14; IgG2a, 80% inhibition at day 7 and 72% at day 14). The increase in the IgM response was also maintained until day 14 (4.8-fold at day 7, 2.3-fold at day 14). Clearly the immunosuppression using sCD40- $\gamma$ 1 is maintained only as long as serum concentrations do not fall below a critical level.

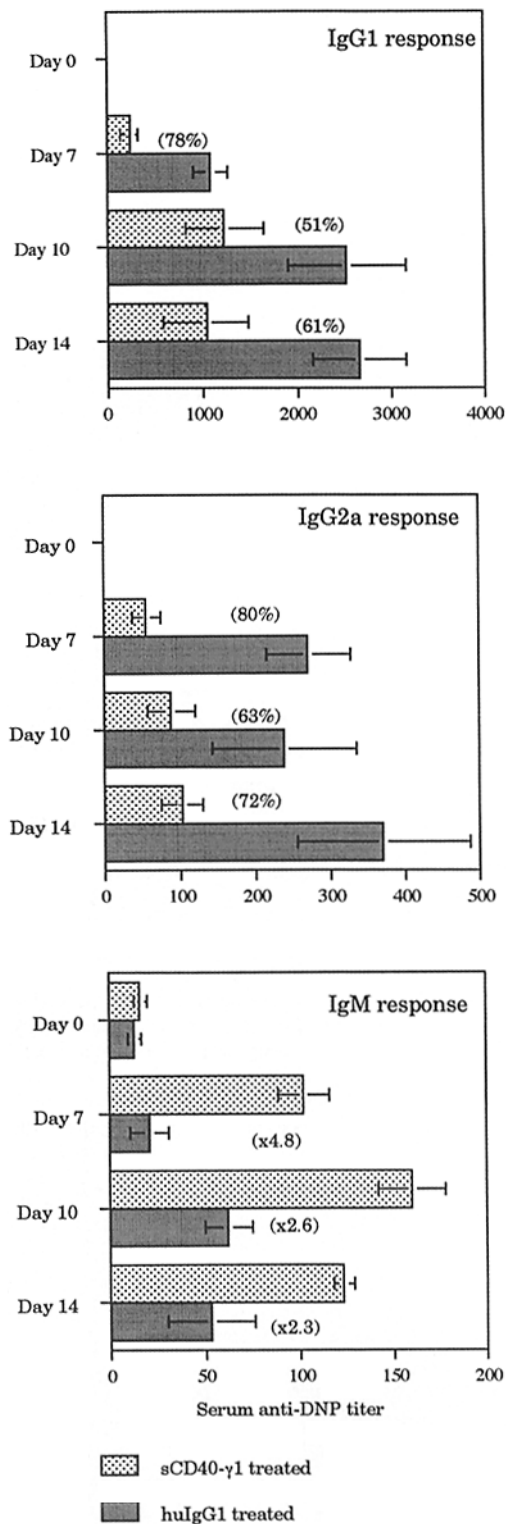
*sCD40- $\gamma$ 1 Does Not Act by Removing Antigen-activated T Cells from the Lymphoid System.* In these experiments, we hope to study the effect of blocking the interaction of CD40 on the B cell with its ligand on specifically activated T cells. The use of a chimeric protein containing the Fc portion of



**Figure 2.** The effect of in vivo administration of sCD40- $\gamma$ 1 on the primary antibody responses to DNP-OVA and C5. Mice were treated with sCD40- $\gamma$ 1 or huIgG1 for the first 5 d of the response or treated with sCD40- $\gamma$ 1 from days 4-8. Serum responses to DNP-OVA in IgG1 (A) and IgM (B) classes and C5 in IgG1 (C) or IgM (D) classes are shown. The results are presented as the reciprocal of the dilution that gave 30% of maximal binding (by standard sera). The mean and standard deviation of titers from four to six mice per group are shown. The figures in brackets show the percent inhibition or fold-augmentation of the sCD40- $\gamma$ 1-treated group compared with the huIgG1-treated group.

Ig in vivo could lead to the killing or removal of CD40-ligand-expressing T cells by a number of means. The first is by complement-mediated killing. This was ruled out in these experiments because we used a strain of mice (DBA/2) that is deficient in C5 due to a widespread genetic defect in mouse populations (47), which prevents activation of the membrane attack complex (C6-9). However, there is still the possibility of opsonization of cells either with complement or the Fc portion of the CD40 chimeric molecule. This may lead to removal either by macrophages or to antibody-dependent cell cytotoxicity. If activated T cells were removed in this way, the number of cells expressing activation markers other than CD40-L should also be depleted in mice injected

with sCD40- $\gamma$ 1 after immunization. The peak of CD25 (IL-2 receptor) expression is around days 2-3 after injection of alum-precipitated antigen (no *Bordetella pertussis*). Given the rapid expression of CD40-L in vitro and the fact that the in vivo effects are only apparent if the sCD40- $\gamma$ 1 is given early in the response, we would expect CD40-L expression in vivo to be earlier than CD25. Fig. 4 A shows that the number of CD4<sup>+</sup> T cells that acquired CD25 during the course of the response was similar in sCD40- $\gamma$ 1-treated mice and in control mice. A second approach was to measure the frequency of antigen-specific T cells in mice treated with sCD40- $\gamma$ 1. Fig. 4 B, a representation of data from a limiting dilution analysis of OVA-specific T cells that produce IL-3 (i.e., both



**Figure 3.** Effect of administration of sCD40- $\gamma$ 1 for the first 10 d of an antibody response to DNP-OVA. Responses to DNP in the IgG1, IgG2a, and IgM classes are measured. The results represent the mean and standard deviation of serum titers from six mice per group. For further details, see the legend to Fig. 2.

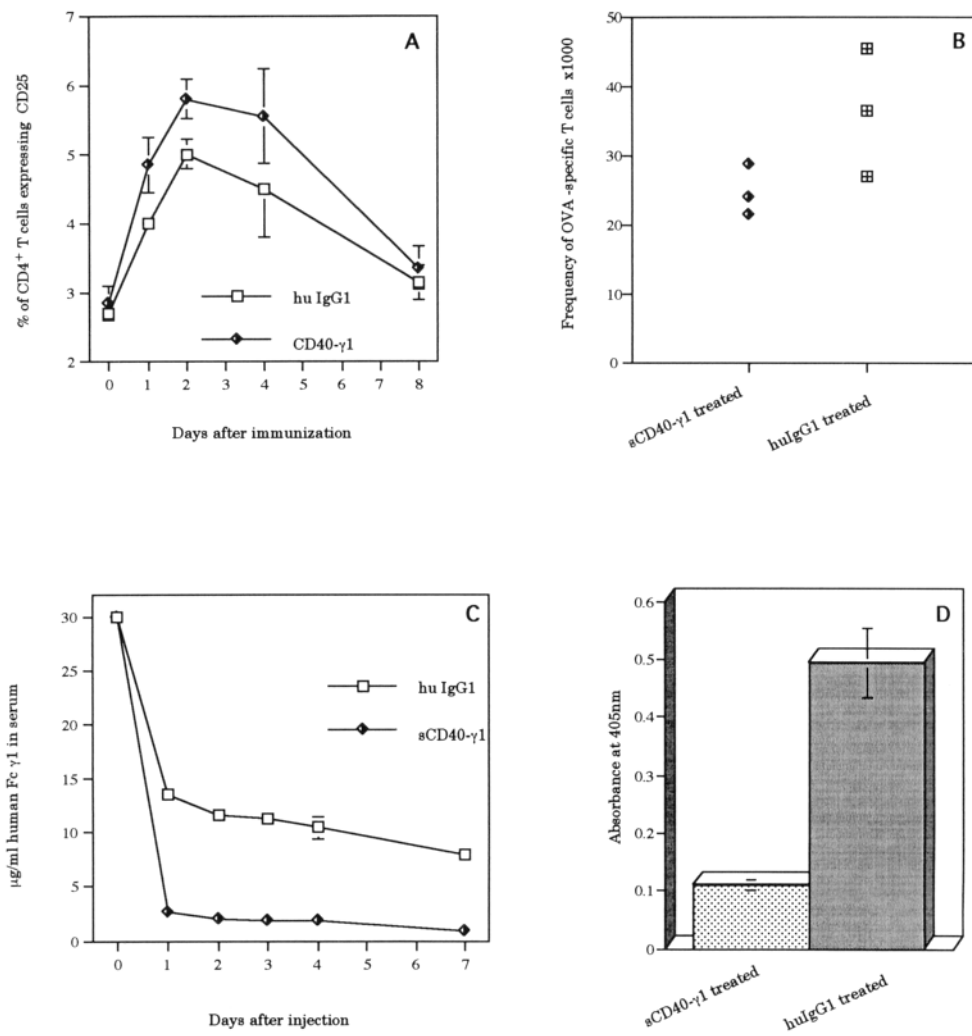
Th1 and Th2), demonstrates that far from a depletion of antigen-specific cells in these mice, the frequency seems to be slightly higher (mean frequency of 1 in 24,000 spleen cells as opposed to 1 in 36,000 in control mice).

Other considerations for the efficacy of sCD40- $\gamma$ 1 in vivo include its half-life and the development of anti-human Fc $\gamma$ 1 responses. Fig. 4 C shows that although a large fraction of the sCD40- $\gamma$ 1 (as well as huIgG1) was cleared from the circulation within 24 h of a single injection, a level of 2  $\mu$ g/ml was maintained for several days. However, higher levels (> 10  $\mu$ g/ml) were maintained in experimental mice that were injected each day. A response to the human Fc $\gamma$ 1 was almost undetectable in mice injected for 5 d with sCD40- $\gamma$ 1, in contrast to those given huIgG1 (Fig. 4 D).

*sCD40- $\gamma$ 1 Has No Effect on Secondary Responses.* Normal mice, immunized 2 mo previously with alum-precipitated KLH, were boosted with 10  $\mu$ g of soluble DNP-KLH. Half of the mice were treated for 5 d with sCD40- $\gamma$ 1 and half with huIgG1. Fig. 5 A shows that there was no appreciable difference between the IgG1 response to carrier, KLH, in either group. In the IgM anti-KLH response (Fig. 5 B) there was a trend towards higher titers in the sCD40- $\gamma$ 1-treated mice, but this is not significant. The response to the DNP hapten is a primary B cell response in which T cell help is not limiting and is provided by memory T cells. Fig. 5 C shows that this response is CD40-L-dependent in that the IgG1 anti-DNP titers were reduced by >50% at both days 7 and 14. In this experiment, the enhancement of the IgM titers was only modest (1.4-fold at day 7; Fig. 5 D).

*sCD40- $\gamma$ 1 Treatment Inhibits the Generation of Memory B Cells.* 10 wk after treatment of DNP-OVA-immunized BALB/c mice (Igh<sup>a</sup>) for 5 or 10 d with sCD40- $\gamma$ 1 or huIgG1, as described, the mice were killed and spleen and peripheral lymph nodes taken. Cell suspensions from similarly treated mice were pooled.  $5 \times 10^6$  cells were injected into lightly irradiated C.B20 mice (Igh<sup>b</sup>) together with  $5 \times 10^6$  cells from KLH-primed C.B20 mice. The mice were immunized with DNP-KLH. In this way we were able to measure the anti-DNP memory B cell response and ensure that T cell help is not limiting. In the first experiment, shown in Fig. 6 A, mice treated during the first 5 d of the primary response exhibited a marked diminution in their memory response (i.e., donor IgG1) 10 wk later. This was especially noticeable after transfer of spleen cells (62% inhibition; with lymph node cells 36% less than controls). Unexpectedly, cells from mice treated from days 4–8 of the primary response with sCD40- $\gamma$ 1 exhibit distinctly enhanced memory response (increased by 70% in the spleen cell transfer and 40% with the lymph node cells). This phenomenon was observed in all three experiments of this type that we performed.

In a similar experiment to the one described, we looked at the development of memory in mice treated with sCD40- $\gamma$ 1 for the first 10 d of the primary response. It was thought that a longer term treatment might have a more profound effect on the memory response as it would cover the time during which GC were developing. Fig. 6, B and C shows the donor-derived memory response of IgG1 and IgM classes.



**Figure 4.** Antigen-activated T cells are not deleted in sCD40- $\gamma$ 1-treated mice. (A) Expression with time after immunization of the activation marker CD25 in sCD40- $\gamma$ 1- and huIgG1-treated groups. (B) Frequency of antigen-specific, IL-3-secreting T h cells in sCD40- $\gamma$ 1- and huIgG1-treated mice as measured by a limiting dilution assay. Each point represents the value obtained from one mouse. (C) Decay over time of sCD40- $\gamma$ 1 and huIgG1 molecules in the serum of mice after a single injection of 100  $\mu$ g. Mean and standard deviations of results from three mice are shown. (D) Anti-human IgG1 response in mice injected with sCD40- $\gamma$ 1 or huIgG1 for 5 d. Serum was assayed when the mice were bled 9 d later. Antibody binding is represented as the absorbance value given by the ELISA reader. Means and SD from 10 such mice are shown.

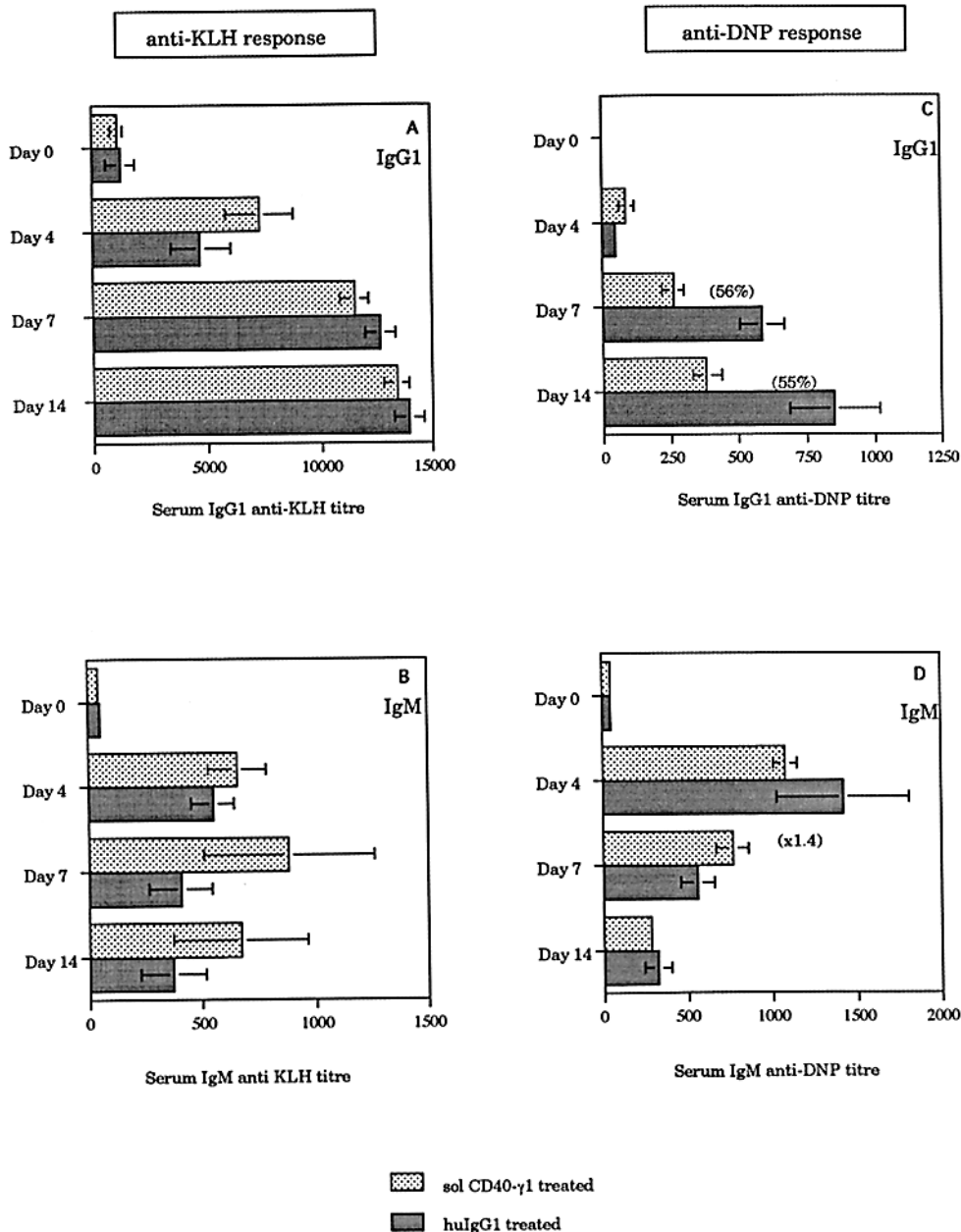
The impairment of the donor IgG1 response was slightly more profound than in the previous experiments (74% of the control responses). It is interesting to note that a small but significant donor-derived IgM response appeared early (by 7 d) in the sCD40- $\gamma$ 1-treated mice but was absent in the control mice. Thus it appears that a small population of IgM-producing memory cells developed in the sCD40- $\gamma$ 1-treated mice which, under normal circumstances, is negligible and hard to detect (Fig. 6 C). It should be noted that this in no way compensates for the impairment in the IgG memory response.

*sCD40- $\gamma$ 1 Treatment Has No Effect on the Generation of Germinal Centers.* Lymphoid tissues taken from mice treated with sCD40- $\gamma$ 1 or huIgG1 for the first 10 d of a response to DNP-OVA were sectioned and examined for the presence of GC by staining with PNA-biotin. Fig. 7 shows that there was no appreciable difference in the number or size of the GC in either group. It was conceivable that as Th cells expanded towards the end of the first week of the response, the efficiency of CD40 blockade was decreased. To address this we looked at the GC reaction in lymph nodes draining a site of footpad

injection of DNP-OVA as the reaction was accelerated in lymph nodes, and was apparent by days 4–5 (48). After immunization, the mice were injected daily with sCD40- $\gamma$ 1 or huIgG1 into the front legs (subcutaneously into the upper surface of the leg). Sections of these axillary and brachial lymph nodes at day 5 revealed no difference in the development of PNA-positive GC (data not shown) between the two groups. The assessment of GC development was possible because we used mice that were bred and maintained, before use, under specific pathogen-free conditions. Such mice have no preexisting GC in the spleen. In addition, unimmunized mice housed in the same cages as the treated mice had no splenic GC (data not shown) and so we are confident that the GC reactions observed were elicited by the immunization.

## Discussion

The available experimental evidence suggests the interaction of CD40 with CD40-L occurs at a variety of levels during T-dependent B cell responses. It is implicated in B cell activation/proliferation (1–5, 16–18), in differentiation to anti-

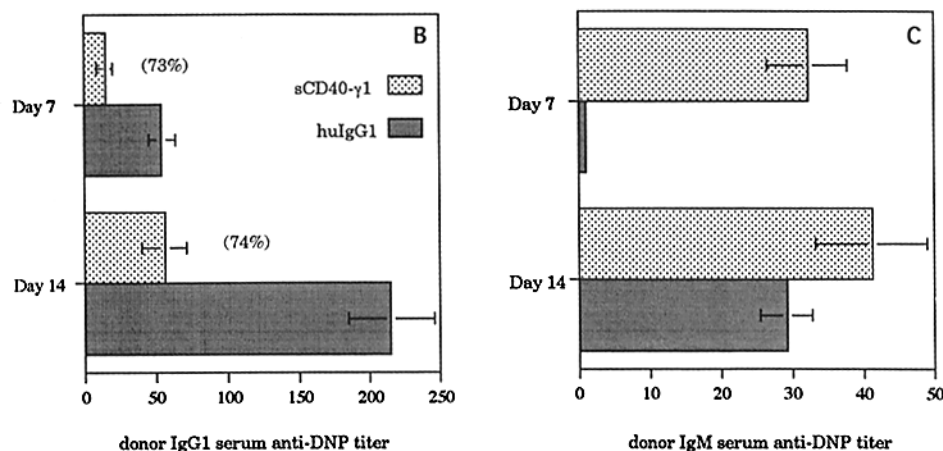
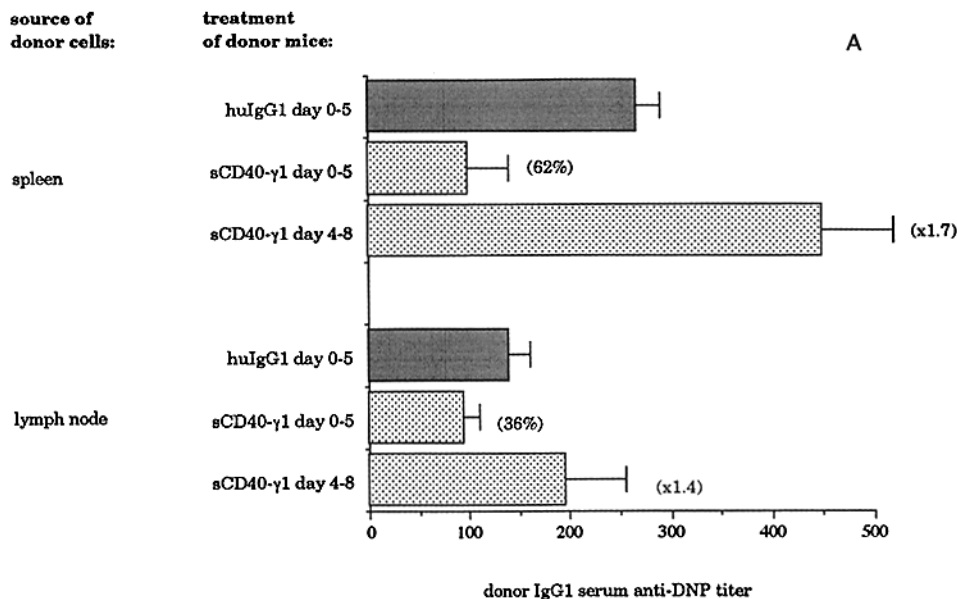


**Figure 5.** sCD40- $\gamma$ 1 has no effect on a secondary anti-KLH response but inhibits the primary response to hapten on the same carrier. Mice, primed 8 wk previously with alum-precipitated KLH were treated with sCD40- $\gamma$ 1 or huIgG1 for the 5 d after boosting of the mice with soluble DNP-KLH. Secondary serum responses to KLH in IgG1 and IgM classes are shown in *A* and *B*, respectively, whereas the primary responses to DNP are shown in *C* (*IgG1*) and *D* (*IgM*). The results are presented as the reciprocal of the dilution that gave 30% of maximal binding (by standard sera). The mean and standard deviation of titers from six mice per group are shown. The figures in brackets show the percent inhibition or fold-augmentation of the sCD40- $\gamma$ 1-treated group compared with the huIgG1-treated group.

body-secreting plasma cells (19–21), in isotype switching (26–29), in rescue/selection of cells in GC undergoing affinity maturation (36, 39), and in the generation of memory B cells (36, 39). The aim of these experiments was to place these multifarious observations into the context of immune responses in vivo. Three major conclusions can be drawn from the results. (a) Memory B cell populations do not develop if the CD40–CD40 ligand interaction is blocked even though GC development appears to be unaffected. This is one of the first direct demonstrations that CD40 signals are necessary for the development of memory B cells. (b) T-dependent antibody responses can proceed in the absence of CD40 ligation, albeit in a quantitatively and qualitatively inferior form. This has recently been the subject of some debate arising from

the analysis of HIMG1 patients. (c) Ligation of CD40 is essential for the maturation of T-dependent antibody response to its full range of isotypes, in agreement with many previous observations, not least HIMG1. The approach of blocking CD40 ligation at defined points of the immune response has clear advantages over analysis of genetic mutants that lack functional CD40 ligand activity from birth. We can investigate the levels of action of what appears to be a multifunctional receptor–ligand interaction, avoiding the potential problem of an immune system that has undergone a dysfunctional development. For instance, HIMG1 patients exhibit severely disorganized secondary lymphoid tissues in which normal cell interactions may be precluded (see below for further discussion).

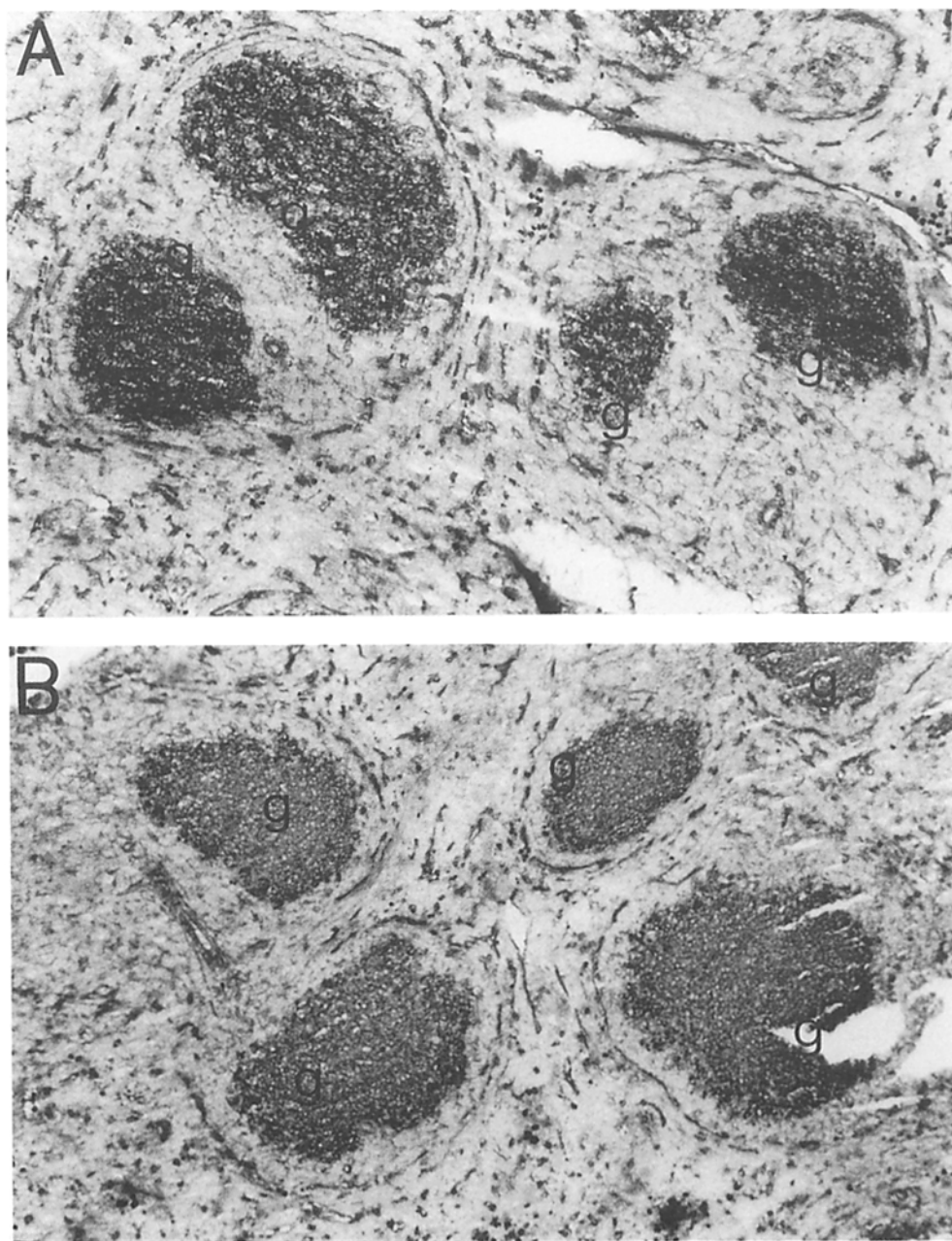




**Figure 6.** The generation of memory B cell populations is impaired in mice treated with sCD40- $\gamma$ 1. (A) Donor IgG1 anti-DNP memory responses in adoptive hosts of cells taken from mice treated 10 wk previously with sCD40- $\gamma$ 1 or huIgG1 for either days 0-5 or 4-8 after priming. Cells from spleen or lymph nodes were assayed separately. B and C show the donor IgG1 (B) or IgM (C) responses derived from cells taken from mice treated with sCD40- $\gamma$ 1 or huIgG1 for 10 d during the primary response 10 wk previously. The mean and standard deviation of titers from five to six mice per group are shown. The figures in brackets show the percent inhibition or fold-augmentation of the sCD40- $\gamma$ 1-treated group compared with the huIgG1-treated group.

**CD40-L-dependent Pathway for T-dependent B Cell Activation.** The most obvious conclusion to be drawn from these data is that, for the responses examined, blocking of the interaction of CD40 with its ligand on activated T cells has profound consequences for both the quantity and quality of the primary antibody response. Daily injections of sCD40- $\gamma$ 1 during the first 5 d after immunization abrogated, to a large degree, the predominant IgG1 response to a variety of antigens. Whereas the IgM response was augmented in comparison with untreated mice, this in no way compensated for the loss of the IgG response. With all of the antigens used, the IgG1 response was at least 10-fold higher than that of the IgM, however, the enhancement of antigen-specific IgM observed in the sCD40- $\gamma$ 1-treated mice was rarely greater than five-fold. Indeed, measurement of the total antigen-specific antibody production (in all classes) demonstrated a reduction in titer of at least twofold in all the experiments.

Given the necessarily crude nature of the in vivo experiment, we cannot know with absolute certainty at which level of the response sCD40- $\gamma$ 1 has its effect. It may interfere with B cell activation and proliferation, isotype switching, or differentiation to antibody-secreting cells. However, there are clues. That the increase in the antigen-specific IgM response does not wholly compensate for the reduction in the IgG subclasses suggests the number of B cells activated or the burst size of the activated clones is smaller in sCD40- $\gamma$ 1-treated mice. If this were not true (i.e., if the clone sizes were similar), the amount of antigen-specific antibody produced (irrespective of class) should be the same in both sCD40- $\gamma$ 1-treated and control groups. This conclusion assumes that there is no difference in the rate at which either activated B cells differentiate into IgM or IgG-producing plasma cells or at which IgM or IgG plasma cells secrete antibodies (there is no evidence to suggest this assumption is incorrect). This



**Figure 7.** GC develop normally in sCD40- $\gamma$ 1-treated mice. Cryostat sections of spleens from mice treated either with sCD40- $\gamma$ 1 (A) or huIgG1 (B) for 10 d after an interperitoneal injection of alum-precipitated DNP-OVA; stained with biotin-labeled PNA that is highly expressed on GC B cells and revealed using horseradish peroxidase-labeled Streptavidin. Darkly stained GC are marked (G).  $\times 100$ .

argument places the “CD40 block” at an early point in the B-T cell interaction that leads to B cell activation and subsequent proliferation. The fact that delaying the onset of administration of sCD40- $\gamma$ 1 had little or no effect on the specific immune response supports this analysis.

There are a number of scenarios that might explain the inhibition of isotype switching brought about by a CD40 block in these experiments or in hyper-IgM patients. (a) The simplest notion, and one that fits with the conclusion that the CD40 block is at the earliest stage of B cell activation, is that isotype switching will not proceed in the absence of extensive cellular proliferation (49). (b) Alternatively, the ligation of CD40 during B cell activation may initiate a cas-

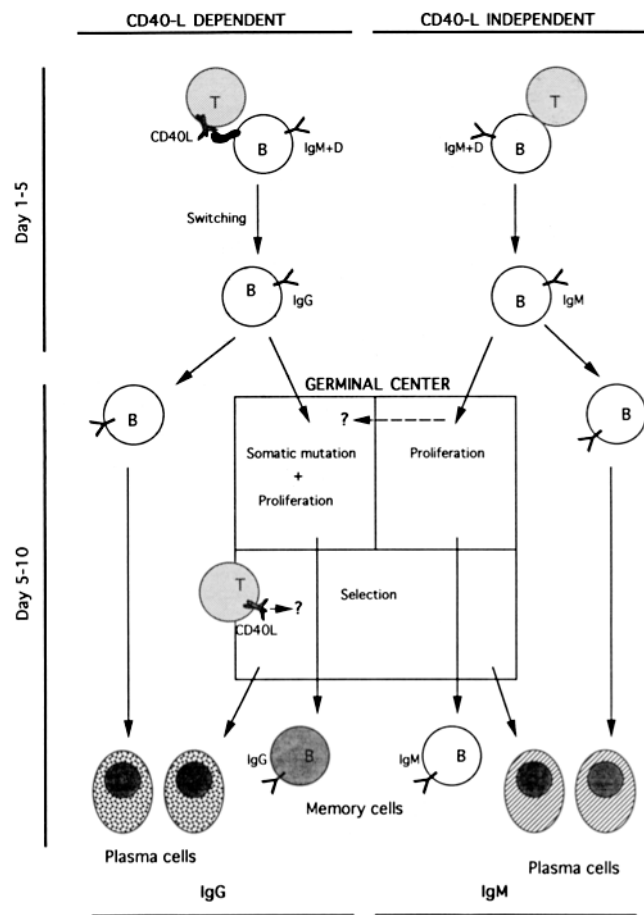
cade of signaling events concluding with the expression of lymphokine receptors, mediating signals that induce isotype switching and terminal differentiation (50). The fact that resting B cells express low levels of IL-4 receptor and are responsive to the lymphokine when delivered in combination with an anti-Ig signal suggests that this proposition is not the whole story. (c) A variation on the second possibility is that the T cell requires the signal via CD40-ligand to proceed to high rate lymphokine production. The current experiments do not allow us to distinguish which of these proposals is correct.

*CD40-independent Pathway of T-dependent B Cell Activation.* In these experiments and in hyper-IgM patients (26–29),

the absence of CD40 ligation does not completely inhibit T-dependent antibody production. The “compensatory” IgM antibody response is T cell dependent. IgM is often viewed as a relatively T-independent antibody class, in that responses to T independent antigens are restricted largely to this class, however, T cell help is required for the IgM responses to TD antigens. The antigens used in this study do not elicit responses in nude, athymic mice (Gray, D., personal observation). Clearly, IgM responses to some antigens are T-dependent and patients with defective CD40-ligand can make responses to TD antigens (21, 31). Thus there is a pathway for T-dependent B cell activation that is independent of CD40-signaling. There is some confusion in the recent literature on this point. Korthäuer et al. (26) report that the T-dependent PWM fails to induce IgM secretion from cells of HIGM1 patients, this only being possible in the presence of cross-linking antibodies to CD40. This directly contradicts a number of older studies (32–34) in which cells from HIGM1 patients produced IgM in response to PWM. It seems likely to us that a variety of signaling pathways are still open to B and T cells in the absence of CD40-L and that these can lead to IgM secretion by the B cell (for a review, see reference 51).

Not only do mice treated with sCD40- $\gamma$ 1 make specific IgM responses, they make super-normal responses. The simplest explanation for this is that cells which would normally be diverted into the “IgG arm” (CD40-L dependent; Fig. 8) of the response by ligation of their CD40 remain, instead, in the IgM arm. Thus, more Ag-specific precursors enter this pathway than would normally do so. As we have already discussed, the fact that this hyper-IgM response does not wholly compensate for the loss of the IgG response may reflect the less intense clonal expansion in the IgM arm (CD40-L independent) in comparison with the IgG arm (CD40-L dependent).

Secondary responses also seem to be relatively CD40-L independent. Treatment of mice with sCD40- $\gamma$ 1 during the 5 d after a boost of KLH has little observable effect on the IgG antibody titers. It might be argued that the larger number of CD40-L-expressing, antigen-specific Th cells present in the secondary response would mean that the blockade of CD40-L would require larger doses of sCD40- $\gamma$ 1 than we used. This does not seem to be the case as in the very same mice a concomitant primary response to DNP helped by the same KLH-specific memory T cells is inhibited by the dose of sCD40- $\gamma$ 1 that we use. In other words preactivated or memory B cells do not rely upon a signal via CD40 to proliferate and produce antibody. This result is in contradiction to that recently published by Foy et al. (52) in which mice are treated with a hamster antibody to CD40-L. Indeed there are a number of points upon which there are discrepancies. Foy et al. (52) find profound and long-term inhibition of primary IgM and IgG responses and secondary responses. All of the effects that we observed are transient except for the impairment of memory development (see below). This might be explained by the longer half-life of their antibody, a higher affinity for the ligand than the natural receptor, higher doses used, or any combination of these. We suspect in addition that such profound and long-term effects indicate a differ-



**Figure 8.** Scheme of B cell differentiation illustrating CD40-ligand-dependent and-independent pathways. Note that the CD40-L-independent pathway seems to include proliferation in GC but may not involve the processes of somatic mutation and affinity selection.

ence in the mode of action between the anti-gp39 antibody and the soluble CD40- $\gamma$ 1; i.e., anti-gp39 (MR-1) causes deletion of T cells that express CD40-L. We have shown by limiting dilution analysis that the frequency of antigen-specific Th cells is not reduced in sCD40- $\gamma$ 1-treated mice. Foy et al. (52) have not made a frequency analysis but use an adoptive transfer system to show that T cell populations from treated mice can support a secondary type response in adoptive hosts. There are two reasons to believe that this control will be inconclusive: First, they transfer a vast excess of primed cells ( $5 \times 10^7$ ). In our own experience of adoptive transfer assays of memory B cells,  $5 \times 10^6$  cells is sufficient and optimal if quantitation is required. A significant reduction in the frequency of antigen-specific T cells in MR-1-treated mice would go undetected in this experiment. Second, the population transferred to the adoptive hosts will contain T cells that have already been activated but which, by day 7 before transfer, may not yet have expressed CD40-L. These will still be capable of supporting the B cell response in the adoptive host.

*Development of Memory B Cells Is Impaired by CD40-L Blockade.* Treatment of mice for even the first 5 d of a re-

sponse with sCD40- $\gamma$ 1 caused a depression of the memory response (by >60%) 10 wk later. The impairment of memory development was increased to >70% by treatment for the first 10 d of the response. The fact that the memory population does not recover with time, like the primary IgG1 antibody response, suggests that the decision to enter the memory B cell pathway is made during the very early stages of the immune response. This notion is supported by the observation that mice, in which the sCD40- $\gamma$ 1 injections were delayed until day 4 of the primary response, develop normal and possibly enhanced memory responses. There are, at least, two ways to explain this phenomenon. First, a particular type of T cell interaction, presumably involving CD40 ligation, is necessary at an early stage to allow the B cell to enter the memory pathway. The alternative is the proposal of Linton et al. (53) that preexisting in the animal is a population of memory precursor B cells that are preferentially utilized in memory responses and not in primary antibody production. If this is the case, the data presented here indicate that this memory precursor population is dependent upon CD40 signaling for clonal expansion. Whichever of these two alternatives is correct, it is clear that the IgM-producing/CD40-L independent arm of the response does not normally give rise to appreciable numbers of memory B cells. However, in sCD40- $\gamma$ 1-treated mice, a small but significant IgM memory response is revealed at day 7 which is undetectable in the control group. We should point out that we cannot conclude from these data that the CD40 signal is wholly sufficient for memory cell differentiation. Other signals may be required; we favor the idea that CD40 ligation induces a state of differentiation in which the cells can receive "survival" signals.

In a number of experiments we have observed that the memory populations in the spleen are more severely affected by sCD40- $\gamma$ 1 treatment than populations recovered from lymph nodes. In the responses examined the major part of the memory response seems to be derived from cells residing in the spleen. The responses from donor spleen cells are up to twofold higher than those achieved by transfer of a similar number of lymph node cells. A discrete population of memory cells is known to reside in the marginal zone of the spleen (54). These cells are not found in significant numbers in lymph nodes. Antigen-specific B cells settle in the marginal zone during the first week after immunization and remain there for several weeks as a pool of cells quite separate from the recirculating pool (marginal zone B cells are sessile; 54). Any bias of the response towards this splenic population may simply reflect the route of immunization (intraperitoneal), although clearly its development seems dependent upon an early CD40 signal. The development of hapten-binding B cells in the marginal zone of animals treated with sCD40- $\gamma$ 1 is now under investigation.

*The Signal for Initiation of GC Proliferation Is Not Delivered via CD40.* The central role of GC in the generation of memory B cells is well established (for a review see reference 55). For this reason we were surprised to find that in sCD40- $\gamma$ 1-treated mice, there was no diminution of the GC reaction, either in size or in the number of foci. Even mice that made virtually no IgG response to the immunizing antigen

(>90% inhibition) exhibited perfectly normal GC development. This might indicate that the concentration of sCD40- $\gamma$ 1 required for blocking of the IgG switch is insufficient to inhibit GC development (because of differences in the affinity or number of interactions necessary). We do not believe that this is the case as neither prolonged injection of 100  $\mu$ g/mouse/d (for 10 d; Fig. 7) nor administration of 500  $\mu$ g/d over 8 d (two mice, data not shown) had any effect on the appearance of GC. This result is in sharp contrast to the study of Foy et al. (56), who show that injection of a hamster antibody to CD40-L ablates GC formation. In our minds the discrepancy is due to the different action of the two treatments. As we have previously discussed we suspect that the hamster antibody has its effects by deleting antigen-specific, activated T cells. At face value, these data also appear to contradict the observation in HIMG1 patients that no GC develop, however, upon further examination, it may not be so unexpected. HIMG1 secondary lymphoid tissues show gross abnormalities that include a lack of B cell follicles (31). It may not be surprising that GC do not form in these people if the basic structures and accessory cells are missing. The signals that initiate GC proliferation and the phenotypic changes associated with GC B cells have been a matter of intense interest in this and several other laboratories for some time. We find that cross-linking of CD40 with CD40-L in vitro does not cause these changes (our unpublished data).

Our preferred conclusion is that CD40 signals are not necessary for the initiation of proliferation of B cells in GC. B cells activated via CD40-L-dependent or -independent pathways are equally likely to enter and proliferate in GC. Under normal circumstances, the dominant GC population would consist of B cells activated in association with CD40-L-bearing T cells because of their greater frequency (arising from their greater proliferative activity; see above). Whereas entry into the GC differentiation pathway and proliferation in GC may be CD40-L-independent, CD40 signaling is thought to play a role in the selection of cells within the GC, as GC B cells in vitro can be rescued from apoptosis by antibodies to CD40 (36). The product of that rescue is a small sIg<sup>+</sup> lymphocyte, leading to the speculation that the CD40 signal induced memory cell differentiation. In one way the current data concur with this conclusion in that even though the GC reaction proceeds in sCD40- $\gamma$ 1-treated mice, there is little generation of memory cells. On the other hand, the crucial CD40 signal may be delivered earlier, before entry into the GC. For instance, a regime of injections of sCD40- $\gamma$ 1 designed to coincide with the early phase of the GC reaction (i.e., days 4–8) has no effect on the development of memory, in contrast to early onset of injections (days 0–5). Also, the most convincing study to date of the localization of CD40-L-expressing T cells in lymphoid tissues places them only in extra-follicular sites (57) where primary activation is thought to occur (58). These data are consistent with the notion that only CD40-ligated B cells have the capacity to be selected for re-entry into the recirculating pool and long-term survival as memory cells.

In the context of events that are happening within the GC,

we should keep in mind that whereas number and size of GC are easy to measure, the quality of the GC in sCD40- $\gamma$ 1-treated mice is more difficult to assess. We are currently looking for the presence of somatic mutations in cells from sCD40- $\gamma$ 1-treated mice and are measuring the affinity of antibodies derived from these cells after fusion to generate hybridomas. The fact that cells in sCD40- $\gamma$ 1-treated mice appear to proliferate in the GC but do not enter the "long-lived" memory pool might indicate that the cells die in situ. Our preliminary analysis of apoptosis in GC cells from these mice indicates a three- to fivefold increase in cell death compared with the same populations from control mice (assessed by titration of GC B cells into a DNA fragmentation assay; data not shown).

In conclusion, all of the data presented indicate that the most important site of action of CD40-L in T-dependent antibody responses is during the earliest phases of B-T cell interaction. All of the downstream effects of the sCD40- $\gamma$ 1 treatment, such as lack of isotype switching, antibody secre-

tion, and memory cell formation can be accounted for by an early block. In addition, if the blockade is delayed by 4 d none of these lesions develops. Fig. 8 summarizes two pathways of B cell differentiation that can be defined based on a dependence on CD40-L. The CD40-L-independent pathway appears to be a minor pathway under normal circumstances, however, it does have the potential (in these experiments or possibly with certain antigens) to generate T-dependent IgM responses and a small IgM memory response. By far the major pathway is CD40-L dependent, leading to the full range and volume of antibody production and to the vast majority of memory B cells. Irrespective of their interaction with CD40-L all B cells activated within a cognate T cell interaction seem capable of proliferating in GC. We are keen to know if the events of somatic mutation and affinity selection that occur in the GC can proceed normally in the absence of CD40 signals, either to initiate mutation or to mediate long-term survival signals.

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The authors thank Drs. Brigitta Stockinger and Tomas Leanderson for valuable discussions and critical reading of the manuscript. We also thank Drs. Peter Lane, Klaus Karjalainen, and André Traunecker for help in the production of fusion proteins.

This work was funded by grants from the Wellcome Trust (035516/2/92) and the Medical Research Council (UK) (G9113770CA).

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Received for publication 23 December 1993 and in revised form 28 March 1994.

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