B Cell Function in Mice Transgenic for mCTLA4-H'y1: Lack of Germinal Centers Correlated with Poor Affinity Maturation and Class Switching Despite Normal Priming of CD4⁺ T Cells

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Summary
This report outlines the B cell phenotype of transgenic mice that overexpresses the mouse CTLA-4-humanγ1 (mCTLA4-H'y1) protein. Despite the fact that these mice prime CD4⁺ T cells (Ronchese, F., B. Housemann, S. Hubele, and P. Lane. 1994. J. Exp. Med. 179:809), antibody responses to T-dependent antigens are severely impaired. In contrast, T-independent responses are normal which suggests mCTLA4-H'y1 does not act directly on B cells, but acts indirectly by impairing T cell help. The impaired antibody defect is associated with impaired class switching, with low total immunoglobulin (Ig)G and antigen-specific IgG responses, and an absence of germinal center formation in spleen and lymph nodes but not gut-associated tissues. The defective germinal center formation is associated with a reduction in the degree of somatic mutation in hybridomas made from transgenic mice in comparison with those made from normal mice. It seems likely that mCTLA4-H'y1 exerts its effect by blocking an interaction between T and B cells that induce T cell help for B cells.

Costimulation through accessory molecules is thought to play a crucial role in lymphocyte activation (1). One critical receptor/ligand interaction is between CD28/CTLA4 (2, 3) on T cells and B7 and related molecules on professional APCs such as dendritic cells and activated B cells (4-7). Soluble forms of CTLA4 inhibit the induction of proliferation in vitro of most CD4⁺ clones (8) and block MLR reactions (9). Furthermore, injection of mouse CTLA4-humanγ1 (mCTLA4-H'y1)¹ has been shown to inhibit T-dependent antibody production in vivo (10), and rejection of nonvascularized xenografts in mice (11), although prolongation of allograft survival has been more modest (12).

To enable long-term studies of the potential of mCTLA4-H'y1 to modulate immune function, we generated transgenic mice that expressed mCTLA4-H'y1 in their serum, at levels similar to or greater than those achieved in earlier studies (5, 7). Whereas T-dependent antibody responses were profoundly inhibited in these animals, we found to our surprise that priming of CD4⁺ cells was not impaired. In fact, paradoxically, hyperimmunized transgenic animals had increased numbers of antigen-specific CD4⁺ cells, shown to be functional by their capacity to mediate the adoptive transfer of an antibody response (13).

To understand the mechanism of antibody suppression we have investigated the transgenic mice more fully. Total Ig and IgG isotype levels, together with T-dependent IgG antihapten responses, are grossly reduced. This is not attributable to a nonspecific effect of the transgene as control transgenic mice make normal antibody responses. T-independent responses are normal, which implies that mCTLA4-H'y1 exerts its effect by blocking T cell help to B cells, not by affecting B cells directly through B7 and other CTLA4 ligands. Germinal center formation is absent, and is correlated with impaired switch to IgG isotypes, reduced somatic mutation and selection, and reduced antibody production.

Materials and Methods

Construction of Chimeric Human IgG1 Molecules. Chimeric immunoglobulin molecules expressing the extracellular portions of the mouse CTLA4 gene (14) or mouse CD40 gene (15) and the human IgG1 constant domains were created as described previously (8, 16).

Production of Transgenic Mice. Transgenic mice expressing mCTLA4-H'y1 and mouse CD40-humanγ1 (mCD40-H'y1) were produced as described (17) by microinjecting XbaI-EcoRV fragments from plasmids expressing the above fusion proteins (16) into...
the pronuclei of fertilized eggs from (C57BL/6 × DBA/2)F1 mice. Transgenic mice were identified by screening for the expression of human IgG1 in the serum. This was achieved using hemagglutination of SRBC coated (using the chromic chloride method) with a mouse mAb to human IgG1 (R102) (Recognition Sciences, Birmingham, UK). Transgenic mice were then backcrossed with homozygous C57BL/6 mice to carry the immunoglobulin A heavy chain.

**Antigens.** TNP-Ficoll was prepared by making the AECM derivative of Ficol 400 (Pharmacia, Uppsala, Sweden) as described by Inman (18). TNP was then conjugated to Ficoll by dissolving AECM-Ficoll in 0.1 M borate buffer (pH 8.4), adding TNP-sulphonic acid, and stirring the mixture for 4 h. The mixture was then extensively dialyzed against PBS. The immunogenicity of this preparation was confirmed by immunizing athymic nude mice (P. Lane, unpublished observations).

2,4-Dinitro-fluorobenzene was conjugated to KLH and BSA (both from Sigma Chemical Co., St. Louis, MO) using standard methods, and the degree of haptenation calculated as described in (19). The succinimide ester of nitrophenol (NP) (ICI, Northwich, UK) was conjugated to BSA in 0.1 M borate buffer, pH 8.4, for various times at room temperature. Aliquots of the mixture were taken at various times and free NP was separated from that conjugated to protein by Sephadex 25 columns preequilibrated with PBS. The succinimide esters of NP and biotin (Sigma Chemical Co.) were also conjugated to human gamma globulin (Sigma Chemical Co.) under alkaline conditions, and then extensively dialyzed against PBS. (4-hydroxy-3-nitrophenyl)-acetyl chicken gamma globulin (NP-CyG) was a kind gift from Professor K. Rajewsky (Institute for Genetics, Cologne, Germany).

**Measurement of Antibody Titer and Affinity by ELISA.** Specific anti-DNP and anti-NP antibody titers were determined by ELISA. Antibody affinities for the hapten NP and DNP were measured according to the method described by Herzenberg et al. (20). DNP9BSA, DNP9BSA, NP9BSA, and NP9BSA were coated onto 96-well plastic plates (Dynatech Laboratories, Inc., Chantilly, VA) at 50 µg/ml hapten-carrier conjugate in 0.1 M borate buffer (pH 8.4) overnight at 4°C. Plates were washed in PBS (pH 7.4) with 0.2% Tween 20 (Sigma Chemical Co.), and then blocked with PBS containing 1% FCS for 1 h at room temperature. Serial dilutions of serum in PBS containing 1% FCS were then applied, and incubated for 4 h at room temperature. After washing, peroxidase-labeled specific goat anti sera directed against individual mouse isotypes (Southern Biotechnology Associates, Birmingham, AL) were added at the dilutions recommended, and binding of specific antibodies was revealed with the peroxidase substrate, ABTS (Boehringer, Mannheim, Mannheim, Germany), diluted in citrate buffer (pH 4.6) as recommended. Plates were read on an ELISA reader at 405 nm.

**Measurement of Mouse Immunoglobulin Subclasses and Human IgG1.** The concentrations of mouse immunoglobulin isotypes and subclasses in the sera of transgenic and control mice were measured using radial immunodiffusion plates (RID; Serotec Ltd., Oxford, UK). Levels of human IgG1 were estimated with RID plates provided by the same manufacturer.

**Immunohistology.** Sections of spleen, lymph node, and Peyer's patch were taken from control and immunized mice and mounted in Tissue-tek embedding compound (Miles Scientific Division, Naperville, IL) 5-µm frozen serial sections were cut and air dried, fixed in acetone at room temperature for 15 min, and stored in air tight containers at -20°C until use.

The expression of the transgene was detected using a biotinylated anti-human IgG antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Germinal center B cells were identified by staining with biotinylated peanut (Arachis hypogaea) hemaggulitin (PNA) (Sigma Chemical Co.). Follicular dendritic cells (FDC) were identified in the sections using the rat anti-mouse FDC mAb, FDC-M1, generated in our laboratory. This was visualized using a mouse F(ab')2 anti-rat IgG (H and L chain-specific) conjugated to peroxidase (Jackson Immunoresearch Laboratories, Inc.). For labeling of NP specific plasma cells, and to detect complexes of NP protein and antibody trapped on FDC's in follicles, sections were incubated with 1 µg/ml of NP-biotin-human-γ-globulin (NP-H3,G) in PBS containing 10 mg/ml of normal human-γ-globulin to block non specific binding. After a 1 h incubation, the sections were washed three times in PBS, and endogenous peroxidase was blocked with PBS containing 0.3% H2O2 and 0.1% sodium azide. After further washing to remove traces of azide, the cells were incubated for 45 min with streptavidin peroxidase complexes (Vectorstain Elite ABC; Vector Laboratories, Inc., Burlingame, CA). All peroxidase reactions were developed using di-amino benzidine (1 mg/ml) (Sigma Chemical Co.) containing 0.1% H2O2 in PBS (pH 7.6). Specificity was checked on sections from nonimmunized controls, which did not bind NP-H3,G. Immune complex trapping was assessed using aggregated rat antibody which binds its Fc portion to receptors on FDC. As for the FDC-M1 mAb, the rat antibodies were visualized using the mouse anti-rat IgG reagent conjugated to peroxidase.

**Cell Fusions.** mCTLA4-Hy1 transgenic mice and normal C57BL/6 were primed with 100 µg NP coupled to NP-CyG in alum. 6 wk later they were boosted with 100 µg NP-CyG i.p., and 3 wk after that received a tertiary immunization with 100 µg i.p. NP-CyG. 4 d after tertiary immunization, spleen cells from individual mice were fused with the myeloma fusion partner, Sp2/0 (21), using polyethylene glycol 1500 (Boehringer Mannheim). Fused cells were resuspended in IMDM supplemented with 2-ME (5 x 10-5 M), 10% FCS, l-glutamine, penicillin, and streptomycin, and plated into six 96-well flat-bottomed plates to which 5 x 104/well peritoneal feeder cells had previously been added. 24 h later, hypoxanthine, aminopterin, and thymidine (HAT) were added. Supernatants from wells were screened for λ-bearing anti-NP antibodies. Positive wells were sub cloned in hypoxanthine and thymidine medium containing supernatant from an IL6-secreting hybridoma to improve cloning efficiency.

**Preparation of RNA, cDNA, PCR Amplification, and Subcloning into pBluescript Vectors.** RNA from cloned hybridomas that secreted λ anti-NP antibodies were prepared as described elsewhere (22). First-strand cDNA was synthesized using a kit (Boehringer Mannheim), and this served as template for subsequent PCR reactions. The primers used to amplify the variable regions of IgM and IgG hybridomas were as described in (23). IgM, 5'(GCCCTCAGCA-GAGAC)3'; IgG, 5'(GGCCAGTGGATAGAC)3'. To amplify the V186.2 and related genes, a primer binding to the 5' region of the mature transcript was created: V, 5'(CCACTCCAGGTCCA)3'. Purified PCR fragments were directly sub cloned into pBluescript vector (Stratagene, Inc., La Jolla, CA). Briefly, the pBluescript vector was digested with EcoRV restriction enzyme (Boehringer Mannheim), and the linearized vector tagged with ddTTP using terminal transferase (Boehringer Mannheim). The purified PCR products were ligated with the ddT-tailed vector preparation with ligase (Boehringer Mannheim) overnight at 16°C, and then transformed into competent *Escherichia coli.*

The insertion of the PCR fragment was confirmed by preparation of plasmid DNA, digestion with enzymes, and agarose gel electrophoresis.

**Sequencing Reactions.** Inserts from positive pBluescript clones were sequenced on both strands using plasmid primers and sequence.
Generation of Mice Transgenic for mCTLA4-Hy1 and mCD40-Hy1. Transgenic mice that expressed soluble mCTLA4-Hy1 and mCD40-Hy1 were created as described in Materials and Methods. We identified three independent founder mice expressing mCTLA4-Hy1. All lines had the same phenotype. The expression of the transgenes is controlled by the mouse immunoglobulin heavy chain core enhancer and kappa promoter (25), and the protein is expressed and secreted principally in B cells and plasma cells, although there is detectable transcription in RNA from thymus (26), suggesting some low level of transcription in T cells. The mouse B cell immune system is not mature at birth but by 6 wk of age stable levels of expression (between 10 and 30 ng/ml) of the transgenic chimeric protein are achieved in the serum.

The capacity of these soluble molecules to bind and interfere with the normal interaction between receptor and ligand is dependent on: (a) the molar concentration of the soluble transgenic protein and the affinity of interaction of the ligand–receptor pair; and (b) accessibility of the soluble competitor to the ligand.

The molar concentrations of free mCTLA4-Hy1 and mCD40-Hy1 are ~10^{-7} M in these transgenic mice. The affinity/avidity of mCTLA4-Hy1 for B7/BB1 is in the nanomolar range (27). We have tested the binding at 37°C of sera from transgenic mice, and have observed that binding to activated B cells expressing B7/BB1 is saturated. In our hands, mCD40-Hy1 binds less well to CD40 ligand (CD40L), at least compared with a decameric form of CD40, hCD40-Hy (28), which probably accounts for the failure of mCD40-Hy1 to block CD40L. In addition, mCD40L is only up-regulated after cognate interactions, so mCD40-Hy1 may have less opportunity to compete with the physiological ligand.

Both mCTLA4-Hy1 and mCD40-Hy1 transgenic mice are healthy; there is no obvious difference from normal mice in their survival and fertility. They are tolerant to the soluble transgene just as they are to their own immunoglobulin. Grossly by FACS® analysis (Becton Dickinson & Co., Mountain View, CA), the proportions of B, CD4^+, and CD8^+ cells are normal in the spleen and lymph node, although spleen and lymph nodes are smaller in mCTLA4-Hy1 transgenic animals (data not shown). The size difference seems to be attributable to the lack of germinal centers in these animals (see later).

Impaired T-dependent but Not T-independent Responses. Groups of mCTLA4-Hy1 (Fig. 1 A) or mCD40-Hy1 (Fig. 1 B) transgenic mice and their nontransgenic littermates were immunized with the T-dependent antigen DNP-KLH, initially in alum, and boosted with soluble antigen at the times indicated by arrows. There was no significant difference between mice transgenic for mCD40-Hy1 and control animals, indicating that the introduction of the transgenic construct did not nonspecifically impair immunoglobulin production. In contrast, mCTLA4-Hy1 transgenic mice made a negligible primary antibody response, and secondary responses were at least an order of magnitude lower than in control animals. This was not because antibody responses are simply slower in mCTLA4-Hy1 transgenic mice, as normal antibody levels were not achieved at longer intervals after immunization nor even after tertiary immunization.

In contrast to T-dependent responses, mCTLA4-Hy1 transgenic mice make normal responses to the T-independent antigen, TNP-Ficoll (Fig. 2 A). Cross-linking surface immunoglobulin, as T-independent antigens might be expected

![Figure 1](attachment:image.png)

Figure 1. Total anti-DNP antibody responses after immunization with DNP-KLH in: (A) mice transgenic for mCTLA4-Hy1 (●) and their control nontransgenic littermates (O); and (B) mice transgenic for mCD40-Hy1 (●) and their control nontransgenic littermates (O). Mice were immunized where indicated by arrows with 100 μg/ml of DNP-KLH. The protein was alum precipitated for initial immunizations; all boosts were with soluble antigen. Results show mean and standard deviation for four mice in each group. Controls were nontransgenic littermates. The results are representative of at least three experiments.
Figure 2. Antihapten isotype levels in mice transgenic for mCTLA4-Hy1 and their control littermates after immunization with: (A) control (O) and transgenic (●) anti-TNP antibody titers after immunization with TNP-Ficoll. Timecourse and levels of IgM and IgG3 anti-TNP antibody at day 7. (B) DNP-KLH as in Fig. 1 a. Distribution of anti-DNP isotypes at day 14, 7 d after secondary challenge, and at day 42, 14 d after tertiary challenge with DNP-KLH.

Figure 3. Total levels of antibody isotypes: (A) mice transgenic for mCTLA4-Hy1 (●) and their control nontransgenic littermates (O); (B) mice transgenic for mCD40-Hy1 (●) and their control nontransgenic littermates (O). Samples were from 12-wk-old mice and were measured by radial immunodiffusion.

to do, upregulates B7/BB1 on B cells (4). These data would suggest that binding of mCTLA4-Hy1 to B7/BB1 exerts its effect by blocking an interaction with T cells rather than by acting directly on B cells.

Total Isotypes and Antihapten Isotypes. mCD40-Hy1 transgenic animals had normal isotype-specific antihapten antibody responses to both T-dependent and T-independent antigens (data not shown). Measurement of isotype specific antihapten antibodies in mCTLA4-Hy1 transgenic mice showed that levels of anti-DNP IgM antibodies overlapped in control and transgenic groups, but the IgG isotypes, IgG1, IgG2b, and IgG3, were ~10-fold lower in transgenic animals (Fig. 2 B). In contrast, levels of IgM and IgG3 anti-TNP antibodies, the principal isotypes elicited by TNP-Ficoll, were comparable (Fig. 2 A).

The decrease in IgG-specific anti-DNP titers was reflected in the total levels of IgG isotypes in mCTLA4-Hy1 transgenic animals, which were again about an order of magnitude lower than controls (Fig. 3 A). Levels of total IgG isotypes were comparable to controls in mCD40-Hy1 transgenic animals (Fig. 3 B).

Impaired T-dependent Antibody Responses Are Not Due to a Direct Effect of mCTLA4-Hy1 on B Cells. One possible explanation for the impaired T-dependent responses in these animals was that mCTLA4-Hy1 co-cross-linked B7 on activated B cells with Fc receptors and specifically downregulated T-dependent antibody responses. To test this we took B cells from transgenic and control mice and stimulated them with either LPS (T-independent stimulus) or a soluble form of CD40L that we have previously generated (16) in the presence or absence of mCTLA4-Hy1 (Table 1). CD40L induces the expression of B7-like molecules on B cells as has been reported by others (6, 29). Neither transgenic or control B cells were inhibited by the presence of mCTLA4-Hy1, suggesting that mCTLA4-Hy1 exerts its effect in vivo by blocking a specific interaction with T cells, and not by co-cross-linking B7 with Fc receptors.

Lack of Germinal Centers and Trapping of Antigen on FDCs. Tissue sections from mCTLA4-Hy1 and mCD40-Hy1 transgenic mice were incubated with antibodies directed against human γ1. Lymph node sections from these mice are shown in Fig. 4, A and B. In tissue from the mCTLA4-Hy1 mice (Fig. 4 A), plasma cells in the medullary areas are labeled (thick arrows) as expected, since they secrete the transgene. Also labeled are dendritic cells in the T cell areas (smallest
Table 1. Proliferation of Lymphocytes as Assessed by Uptake of \[^{3}H\]Thymidine

| Animal          | mCTLA4-Hy1 | Medium alone | LPS        | CD8\alpha-mCD40L |
|-----------------|------------|--------------|------------|------------------|
| Control 1       | –          | 7,385        | 146,765    | 50 μg/ml         |
|                 | +          | 4,651        | 145,395    | 40,489           |
| Control 2       | –          | 4,081        | 138,506    | 37,121           |
|                 | +          | 3,791        | 127,880    | 32,643           |
| Transgenic 1    | –          | 2,147        | 84,376     | 26,113           |
|                 | +          | 1,712        | 85,556     | 29,078           |
| Transgenic 2    | –          | 1,832        | 86,858     | 28,757           |
|                 | +          | 2,452        | 110,304    | 48,443           |

200,000 lymphocytes/well in a 96-well plate from control littermates or mCTLA4-Hy1 transgenic mice were cultured for 3 d in the presence of medium alone, LPS (50 μg/ml), or mCD40L-mCD8α in combination with 1% supernatants from IL-4-, IL-5-, and IL-6-secreting clones. Stimulations were done in the absence and presence of mCTLA4-Hy1 (25 μg/ml, final concentration). 1 μCi of \[^{3}H\]thymidine/well was added for the last 16 h of culture. All assays were done in triplicate. Variation between triplicate wells was <10%.

arrows) and FDC in the primary follicles (long arrows). Tissue from mCD40-Hy1 transgenic mice (Fig. 4 B) also showed plasma cell labeling (not seen in this micrograph) but none associated with accessory cells in the germinal center (gc) or paracortex. The monoclonal antibody FDC-M1 revealed that the FDC network was less extensive in mCTLA4-Hy1 transgenic animals (Fig. 4 C) in comparison with nontransgenic mice (Fig. 4 D).

The most striking abnormality in the mCTLA4-Hy1 transgenic mice is the lack of germinal centers in their spleens and lymph nodes. The absence of germinal centers was shown histologically using sections of tissue labeled in vivo with bromodeoxyuridine (data not shown), or stained in vitro with PNA (F = primary follicle; Figs. 4, A and C and 5, A and C). Even after four immunizations with either DNP-KLH or NP-C3\'G, only occasional very small foci of proliferation could be identified in mCTLA4-Hy1 transgenic mice. This was in marked contrast to the mCD40-Hy1 and control animals in which germinal centers (gc; Figs. 4 B and 5) developed normally. Fig. 5 A shows the presence of anti-NP producing plasma cells in NP-C3\'G immune mCTLA4-Hy1 mice and the absence of NP-immune complex localization on FDC in the adjacent primary follicle. Tissue from control mice (Fig. 5 B) localized the NP-containing immune complexes on FDC, formed germinal centers, and developed NP-specific plasma cells. In contrast to the spleen and lymph node, gut-associated lymphoid tissue from the mCTLA4-Hy1 transgenic mice had normal germinal centers and levels of serum IgA were elevated.

A second marked anomaly in the mCTLA4-Hy1 transgenics is their failure to localize immune complexes (Fig. 5 C). This may be due to the low levels of IgG isotypes in their serum, as IgG complexes have been shown to localize much better than those of IgM (30). However, passive immunization of mCTLA4-Hy1 transgenic mice with high titer anti-DNP IgG antibody followed by immunization with DNP-KLH did not restore germinal center formation, whereas control mice responded normally (data not shown). gc formation (Figs. 4 B and 5 D) and immune complex localization (Fig. 5 D) were normal in mCD40-Hy1 transgenic mice.

Lack of Selection and Reduced Somatic Mutation in mCTLA4-Hy1 Transgenic Mice. In view of the absence of germinal centers, we tested for somatic mutation in the mCTLA4-Hy1 transgenic mice. In C57BL/6 mice the primary T-dependent response to NP is dominated by antibodies expressing a particular V\_n gene, 186.2, in association with λ1 light chains, and this subset of antibodies is also evident in secondary and tertiary immune responses to NP (31). We therefore hyperimmunized mCTLA4-Hy1 transgenic and control mice with NP-C3\'G to maximize the chance of inducing somatic mutation. 4 d after the tertiary boosting, fusions were made in the standard manner. Positive hybridomas were identified by binding to NP and by their expression of X light chains. There were several pronounced differences between control and mCTLA4-Hy1 transgenic mice. First, the frequency of hybridomas was much higher in normal mice. We characterized 11 heavy-bearing anti-NP hybridomas from a normal mouse. 10 of these mAbs were γ1 isotype and one expressed γ2a. From 2 mCTLA4-Hy1 transgenic mice, we found 10 hybridomas. Six hybridomas expressed IgM heavy chain isotype, three IgG3, and only one IgG1. We sequenced six of these clones and compared them with three sequences from the normal mouse. The sequences of the V\_n regions of these hybridomas, are shown in Fig. 6.

The three hybridomas shown from normal mice (C039, C041, and C043) all express V\_n186.2 heavy chains, together with the DFL16.1 D segment and JH2. All three hybridomas have extensive somatic mutations in their V regions (Fig. 6 A). In particular all have a mutation at amino acid position 33, encoding a change from tryptophan to threonine. This mutation has been shown to increase the affinity for NP 10-fold (31). They have different NH\_2-terminal diversity...
Figure 4. Light micrographs of lymph nodes from transgenic and control mice (×80). (A) Immunoperoxidase labeling with antibodies against human γ1 in mCTLA4-Hy1 transgenic mice showing staining of plasma cells (thick arrows), which produce the transgenic protein, the FDC network (long arrows), and dendritic cells in the paracortex (small arrows). (B) Immunoperoxidase labeling with antibodies against human γ1 in the control mCD40-Hy1 transgenic mice show no such localization of the transgenic product on FDC or dendritic cells, although plasma cells producing the mCD40-Hy1 are present in the medullary region but are not seen in this micrograph. (C) Labeling of the FDC network with FDC-M1 in mCTLA4-Hy1 mice. (D) Labeling of the FDC network with FDC-M1 in nontransgenic controls.
Figure 5. Light micrographs showing NP-specific antibody and aggregated antibody localization in tissue from transgenic or control mice (×80). (A) Immunoperoxidase localization of anti-NP producing plasma cells (dark brown) in the spleen of immune mCTLA4-Hy1 mice are seen as three clusters adjacent to a nonlabeled follicle (f). (B) The spleen from a nontransgenic immune mouse prepared at the same time point post immunization as seen in A, contains not only the NP-specific antibody producing cells (dark brown) but also localizes the NP-containing immune complexes on FDC (light brown) and forms gc's. (C) Micrograph shows lack of ability of FDC from mCTLA4-Hy1 mice to trap antibody aggregates. (D) Localization of antibody aggregates by FDC in control mCD40-Hy1 transgenic mice.
The sequences of the germline V, genes (V186.2, J00534, X02066, M15228, and M64142). V186.2 encodes the classical anti-NP heavy chain.

**Figure 6.** (A) Nucleotide sequences of the V, genes expressed in the hybridomas of C and T (transgenic) mice.

**A V186.2 sequences obtained from transgenic (T) and control (C) mice**

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| CDRI1 |
|-------|
| V186.2 |
| T059 |
| C043 |
| T093 |
| C043 |
| T099 |
| C041 |
| T099 |
| C043 |
| T099 |

**non classical anti-NP genes from transgenic mice**

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| J00534 |
|-------|
| T077 |
| J00534 |
| T093 |
| J00534 |
| T099 |
| J00534 |
| T210 |

**B DJ sequences obtained from transgenic (T) and control (C) mice**

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**Figure 6.** (A) Nucleotide sequences of the V, genes expressed in the hybridomas of C (control) 39, C41, C43, and T (transgenic) 77, T93, T99, T142, T210, and T215. Mice were primed and boosted twice, the last immunization 4 d before fusion (see Materials and Methods). Also shown are the sequences of the germline V, genes (V186.2, J00534, X00160, X02066, M15228, and M64142). V186.2 encodes the classical anti-NP heavy chain variable region associated with lambda in C57BL/6. The other genes are coded by their accession numbers for the EMBL database. Underlined are
of their D segments indicating that they are not clonally related.

Only one hybridoma, clone T099, from the mCTLA4-Hy1 mice expressed V\textsubscript{\textalpha}186.2 in association with DFL16.1 and JH2. The V\textsubscript{\textalpha} of this hybridoma was unmutated. The 3' end of the DJ border shows no N sequence diversity, a characteristic feature of primary response hybridomas to NP (31). The five other hybridomas expressed different V\textsubscript{\textalpha} genes. Clone T077 expressed V\textsubscript{\textalpha}23, a V\textsubscript{\textalpha} gene identified in the T-independent response to NP-Ficoll (32). Clone T093 expressed V\textsubscript{\textalpha}124 (33), a V\textsubscript{\textalpha} gene closely related to V\textsubscript{\textalpha}186.2 but expressed by the BALB/c Ig\textsuperscript{\textbeta} immunoglobulin haplotype in the response to NP. The mCTLA4-Hy1 mice were generated in (DBA/2 \times C57BL/6)F\textsubscript{1} mice and although they have been backcrossed onto C57BL/6 and are homozygous at the MHC, they may still carry genes from the DBA/2 background. DBA/2 express the IgH\textalpha haplotype, of which V\textsubscript{\textalpha}124 is a member. Clone T142 expresses V\textsubscript{\textalpha}102.1, a V\textsubscript{\textalpha} gene closely related to V\textsubscript{\textalpha}186.2 (34). Clone T210 expresses a V\textsubscript{\textalpha} gene expressed in the anti-DNP response in association with \lambda light chains (35). Clone T210, like T077, and T093 express a proline (CCT) at position 7, a distinguishing feature of C57BL/6 NP\textsuperscript{\textbeta}-related genes (36).

All clones but one, from either transgenic or control mice, use DFL16.1 in association with either JH4 (three cases) or JH2 (five cases). The exception is hybridoma T093, which uses V\textsubscript{\textalpha}124, the IgH\textalpha V\textsubscript{\textalpha}186.2 equivalent gene, in association with DSP 2.2 and JH3.

While all control hybridomas are extensively somatically mutated, there are only four mutations in the transgenic hybridomas, clones T093 and T142. Because the V\textsubscript{\textalpha}186.2 gene family is so large, it is difficult to be sure that these are not closely related germline genes (32). However, we also sequenced the \lambda light chains (data not shown). The three normal hybridomas had five mutations in the V\textsubscript{\textalpha}, whereas only one mutation was detected in the six transgenic hybridomas. Hybridoma T099 had no light chain mutations.

We made a comparative estimate of the affinities of the hybridomas by the method of Herzenberg et al. (20). The binding of T099 represents the affinity of the germline V\textsubscript{\textalpha}186.2. As has been observed previously, the high affinity hybridomas, C039, C041, and C043, from the normal mouse bound at least as well to the low haptenated protein NP\textsubscript{\textalpha}BSA in ELISA compared to NP\textsubscript{\textbeta}BSA, whereas T099 bound less well to NP\textsubscript{\textbeta}BSA than NP\textsubscript{\textalpha}BSA. The pattern for the other transgenic derived hybridomas was qualitatively the same as T099, suggesting they had lower affinities for NP than the control derived hybridomas (data not shown).

Discussion

This paper investigates in detail the defective antibody production in mCTLA4-Hy1 transgenic mice. In many respects these transgenic mice look phenotypically like athymic nude mice. They have levels of IgM antibody within the normal range, but IgG isotypes are reduced, and they make normal TI-2 antibody responses. Unlike athymic mice, however, they make a T-dependent response to protein antigens, but with a greatly reduced IgG titer. To rule out the possibility that the defects in antibody production might be attributable to the expression of the transgene in B cells and plasma cells, we expressed mCD40-Hy1 as a transgene. The mCD40-Hy1 transgenic mice make antibody responses indistinguishable from those in normal mice. Another possibility is that the transgenic protein binds to CTLA4-ligand expressing cells which are then removed either by an interaction with complement or by antibody-dependent cell-mediated cytototoxicity. We think this unlikely as the dendritic cell architecture in T cell areas, where B7 and related molecules are constitutively expressed, looks quite normal. In addition it seems unlikely that mCTLA4-Hy1 specifically downregulates B cell responses by co-cross-linking CTLA4-ligands and Fc receptors on B cells, as mCTLA4-Hy1 did not reduce the proliferation of B cells stimulated with soluble CD40L, a T-dependent stimulus that induces the expression of B7.

The functional defect in antibody production in mCTLA4-Hy1 transgenic mice is correlated with profoundly impaired gc formation, the cardinal feature of T-dependent antibody responses. gc's are locations where a few B cell precursors (37) proliferate at enormous rates (38) after immunization with T-dependent antigens. At least one of the important functions of gc's, therefore, is clonal amplification. Second, it is clear that germinal centers are sites where immunoglobulin class switching can occur. Follicular B cell blasts, the immediate precursors of the gc reaction, express IgM. Centrocytes, the progeny of germinal center centroblasts, almost invariably express IgG and IgA isotypes (39). In addition, it has been proposed that somatic mutation is specifically switched on in B cells here (40), and the accumulation of somatic mutations in germinal centers has been elegantly confirmed using microdissection and PCR (41). Finally, it is considered likely that long-term antibody responses are maintained by the continuous stimulation of B cells with antigen retained on FDCs (42). With respect to these aspects of gc function, mCTLA4-Hy1 transgenic mice, with no gc's, behave as one might predict. They make poor responses to protein antigens, and have limited class switching. Furthermore, analysis of V\textsubscript{\textalpha} gene usage in the anti-NP response indicates
that the transgenic mice are defective in the clonal expansion of B cells with high affinity for antigen. As in the T-independent response to NP (32), many $V_N$ genes closely related to $V_N$186.2 but of low affinity are represented.

B cells are activated initially in the T cell areas in T-dependent responses (39, 43, 44) where CTLA ligand is expressed on interdigitating cells (5, 8). It is possible that some primary signal is blocked at this location in mCTLA4-Hγ1 transgenic mice. However, staining of sections from mCTLA4-Hγ1 transgenic mice showed localization of CTLA4 ligand on the FDC network, where it may have been trapped nonspecifically from degraded B cells as has been shown for class II molecules (45). Alternatively, FDCs bearing CTLA4 ligand may form a local environment where trapped antigen, B cells, and the many T cells located in this part of the gc interact.

The earliest phase of the follicular reaction involves proliferation of slg-positive cells that have not switched (39). This stage appears to be profoundly blocked in mCTLA4-Hγ1 transgenic mice. The failure of these mice to class switch is reflected in the transgenic hybridomas. Virtually all hybridomas from normal mice express γ1, whereas in transgenic animals $\gamma_2$ and $\gamma_3$ were the predominant isotypes, a profile more usually associated with T-independent responses (46). It is emphasized that gut-associated lymphoid tissue is not affected in the transgenic mice. There is no obvious reason for this as the transgenic protein is expressed in gut-associated plasma cells.

Compared with normal mice, mCTLA4-Hγ1 transgenic mice show reduced levels of somatic mutation. This is consistent with gc's being the principal site of somatic mutation, although a few mutations were observed in the transgenic hybridomas. What is clear from these studies is that the affinity of the hybridomas generated in mCTLA4-Hγ1 transgenic mice is low. This is partly attributable to a lack of ability to selectively amplify B cells encoding high affinity germline genes. Somatic mutation is seen in $V_N$ genes in lower animals like Xenopus, which nevertheless have poor affinity maturation (47). It has been speculated that the failure of Xenopus to make high affinity antibodies is caused by an inability to select mutants rather than a failure to generate them by somatic mutation. mCTLA4-Hγ1 transgenic mice fail to localize immune complexes on FDCs and hence, in addition to their other defects, fail to provide an appropriate microenvironment in which selection of high affinity B cells can take place.

There are striking similarities between these mice and human patients with an antibody deficiency associated with hyper IgM that have a defect in the expression of CD40L on T cells (48-50). Both have low IgG levels, impaired T-dependent antibody responses, and an absence of gc formation. Triggering of human T cells through CD3 is very poor at inducing the expression of CD40L (28). However, it has recently been reported that B7/BB1 in conjunction with an anti-CD3 signal strongly upregulates CD40L (51). The defects in mCTLA4-Hγ1 transgenic mice could be explained by a failure of CTLA4 ligand expressing cells to upregulate CD40L on activated T cells, which consequently fail to supply help for B cells.

In summary, this report confirms and extends previous studies (10) showing that mCTLA4-Hγ1 can inhibit T-dependent antibody responses. It is not yet clear whether established antibody responses are similarly dependent on CTLA4 ligand. Enthusiasm for mCTLA4-Hγ1 as a tool for suppressing T-dependent antibody responses must be tempered by the fact CD4$^+$ T cells are primed normally. It is now clear that there are many ligands for CTLA4/CD28 (6, 7, 52). Differential sensitivity of these costimulatory molecules to the blocking effects of mCTLA4-Hγ1 would readily explain the apparent paradox that CD4$^+$ cells are primed normally in the transgenic mice described here.

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