Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1018–1022, February 1989
Immunology

Major histocompatibility complex class I-specific cytolytic T cells, derived from *gld* mice, lacking Thy-1, CD4, and CD8

(T-cell receptor/T-cell development)

SCOTT WADSWORTH, KATSUYUKI YUI, AND MARK I. GREENE

Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104-6082

Communicated by Bernard N. Fields, November 3, 1988 (received for review October 4, 1988)

ABSTRACT **Thy-1 is a major cell surface molecule expressed on murine thymocytes and peripheral T cells. Its physiological function is unknown, but *in vitro* studies suggest that Thy-1 may transmit activation signals to T cells and may play a role in the growth and/or differentiation of thymocytes [Kroczek, R. A., Gunter, K. C., Seligmann, B. & Shevach, E. M. (1986) *J. Immunol.* 136, 4379–4384; Kroczek, R. A., Gunter, K. C., Germain, R. N. & Shevach, E. M. (1986) *Nature (London)* 322, 181–184]. However, not all mouse thymocytes are Thy-1$^+$ [Scollay, R., Wilson, A., D'Amico, A., Kelly, K., Egerton, M., Pearse, M., Wu, L. & Shortman, K. (1988) *Immunol. Rev.* 104, 81–120]. In addition, C3H-*gld/gld* mice accumulate large numbers of Thy-1$^-$ (and Thy-1$^+$) T-cell antigen receptor-positive CD8$^-$ CD4$^-$ (double negative) T cells in peripheral lymphoid organs. Our previous studies of these Thy-1$^-$ and Thy-1$^+$ double negatives suggested that lack of Thy-1 expression correlated with diminished capacity to respond to T-cell stimuli. In this report, we describe a Thy-1$^-$ α/β T-cell receptor-positive major histocompatibility complex-specific cytotoxic T-cell clone derived from C3H-*gld/gld* lymph node-residing cells. The data show that, at least in this system, Thy-1 (and CD8/CD4) expression is not required for growth, cytolytic activity, or expression of functional T-cell receptor complexes *in vitro* and raise the possibility that Thy-1 expression may not be obligatory *in vivo* for development of cytotoxic T-lymphocyte precursors in *gld* mice.**

The murine Thy-1 molecule is one of the major cell surface glycoproteins expressed on thymocytes, peripheral T cells, certain dendritic epidermal cells (1), and neurons (2). Thy-1 is expressed very early in T-cell development, possibly prior to the emigration of bone marrow-derived pro-T cells to the thymus (3). Thy-1 is present on fetal mouse thymocytes at least as early as day 14 of gestation, preceding expression of the α/β T-cell antigen receptor (TCR) and the T-cell accessory molecules CD8 and CD4 by 2–3 days (4). The *in vivo* function of Thy-1 is unknown. *In vitro*, anti-Thy-1 monoclonal antibodies (mAbs) induce proliferation and lymphokine secretion in mature T cells (5) or thymocytes (6) and induce increased levels of intracellular calcium in T cells, thymocytes, and Thy-1-transfected B cells (7, 8). Full activation of T cells by Thy-1 requires co-expression of CD3/TCR complexes, whereas increases in intracellular calcium can be induced in CD3/TCR$^-$ T cells (9). These results suggest a role for Thy-1 in signal transduction and T-cell activation (8). Interaction of Thy-1 with a putative ligand in the thymus could play a role in triggering the growth and/or differentiation of thymocytes (7, 9).

C3H mice homozygous for the *gld* mutation (C3H-*gld/gld*) exhibit an age-dependent lymphadenopathy accompanied by symptoms of autoimmunity (10). Peripheral lymph nodes in these mice become greatly enlarged, due to the accumulation of large numbers of Thy-1$^+$ CD8$^-$ CD4$^-$ (double negative) and Thy-1$^-$ CD8$^-$ CD4$^-$ surface immunoglobulin-negative "null" cells (10, 11). Most or all of these cells express TCR complexes on the cell surface (12, 13), suggesting they are in the T-cell lineage. Freshly isolated populations respond poorly to conventional T-cell stimuli *in vitro* (11, 12) and exhibit CD3 phosphorylation patterns distinct from those of normal resting T cells (14). Our previous studies found that responses to Con A and alloantigen were more limited in gld Thy-1$^-$ T cells compared to their Thy-1$^+$ counterparts, suggesting that Thy-1 was important for lectin and TCR-mediated responses in these cells (15).

The origin and developmental pathway of these Thy-1$^-$ and Thy-1$^+$ CD8$^-$ CD4$^-$ subsets in *gld* mice is unknown. They may be expanded populations of rare T cells present in normal mice at a particular stage of differentiation or activation. Alternatively, they may be abnormal cells that arise due to an intrinsic defect that alters their development. Based on analyses of TCR gene rearrangement and mRNA expression, we have suggested (13) that these subsets may arise due to aberrant or incomplete intrathymic differentiation, possibly by escaping normal selection mechanisms in the thymus. We have described the cellular and molecular properties of *gld* double-negative and null cell populations (13, 15) and the generation of Thy-1$^+$ CD8$^-$ CD4$^-$ allospecific cytotoxic T-cell clones from C3H-*gld/gld* lymph node (16). In this report, we discuss the growth and response features of *gld* null cell populations and describe in detail a Thy-1$^-$ CD8$^-$ CD4$^-$ CD3$^+$ cytotoxic T-cell clone that expresses an α/β TCR and mediates allospecific cytotoxic activity typical of conventional peripheral T cells, despite its unconventional phenotype.

MATERIALS AND METHODS

Cell Culture. Clones were maintained by weekly stimulation in 12-well culture plates at 5×10^4 cells per ml, with γ-irradiated (2000 rad; 1 rad = 0.01 Gy) C57BL/6 (B6) spleen cells (1.5×10^6 cells per ml) plus 10% (vol/vol) supernatant from Con A-activated rat spleen cells, in a humidified atmosphere of 8% CO_2/92% air at 37°C.

Flow Microfluorimetry. Cultures were harvested 5 or 6 days after stimulation. Viable cells were isolated by Ficoll/Paque density gradient centrifugation, washed, and suspended in 0.5% bovine serum albumin/0.1% sodium azide/phosphate-buffered saline. Fifty microliters containing $>2 \times 10^5$ cells was incubated with anti-Thy-1 (30.H12), anti-CD8 (3-155), anti-CD4 (GK 1.5), anti-TCR $V_{\beta 8}$ (F23.1), anti-K^kD^k (15.3.1), anti-lymphocyte function-associated antigen 1 (LFA-1) (FD441.8), anti-interleukin-2 (IL-2) receptor

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TCR, T-cell antigen receptor; IL-2, interleukin 2; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; CTL, cytotoxic T lymphocyte; LFA-1, lymphocyte function-associated antigen 1.

(3C7), anti-CD3 (2C11), or anti-B220 (RA3-3A1/6.1) mAbs for 20 min on ice. After washing once, cells were stained with either 50 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rat κ chain mAb (Becton Dickinson), 50 μl of FITC-conjugated goat anti-mouse immunoglobulin [$F(ab')_2$ fragment, Cooper Biomedical; with F23.1, 15.3.1], or FITC-conjugated rabbit anti-hamster immunoglobulin (Organon Teknika-Cappel; with 2C11) for 20 min on ice. Cells were washed four times in staining buffer and analyzed on a FACS IV (Becton Dickinson). Anti-B220 was used as a hybridoma-conditioned culture supernatant at a final concentration of 50% (vol/vol). All other antibodies were hybridoma culture supernatants enriched for immunoglobulin by ammonium sulfate precipitation and used at a final dilution of 1:50.

Northern Blot Analysis. Ten micrograms of total RNA was extracted from cells by using the guanidine isothiocyanate method and separated on 1% agarose gels containing 6% (vol/vol) formaldehyde and ethidium bromide at 0.1 μg/ml as described (16). RNA was then transferred to nylon membranes (GeneScreen*Plus*, New England Nuclear). Membranes were prehybridized for 20 min at 42°C in 25 ml of 50% (vol/vol) deionized formamide/1 M NaCl/10% (wt/vol) dextran sulfate/1% $NaDodSO_4$. ^{32}P-labeled cDNA probes were prepared by primer extension as described by the manufacturer of the kit (Pharmacia) and used at 10^7 cpm per blot. Hybridizations were conducted for 20 hr at 42°C with shaking. Membranes were then washed several times as described (16) and autoradiographed at −70°C with intensifying screens.

Immunoprecipitation. Cells were harvested 5 days after stimulation and separated from nonviable stimulator cells by centrifugation on Ficoll/Paque. Surface proteins ($>10^7$ cells) were labeled with ^{125}I by using the lactoperoxidase-catalyzed iodination method (17). Cell lysates were prepared in 1% digitonin buffer (18) and precleared with protein A-agarose and fetal calf serum overnight. Supernatants were then treated with either culture medium or anti-CD3 (2C11) for immunoprecipitation and incubated in the presence of protein A-agarose for 1 hr at 4°C. Samples were washed extensively in digitonin buffer and subjected to polyacrylamide gel electrophoresis under nonreducing or reducing conditions using a 12% running gel. Gels were stained with Coomassie blue, destained, dried, and autoradiographed.

Cytotoxicity Assays. Cytotoxicity was measured by using a standard chromium-release assay. Clones were harvested 4–9 days after stimulation, washed, and resuspended in an appropriate volume of culture medium [RPMI 1640/10% (vol/vol) fetal calf serum/10 mM Hepes/2 mM glutamine/1 mM sodium pyruvate/0.1 mM nonessential amino acids/50 μM 2-mercaptoethanol/1% penicillin plus streptomycin]. Con A-blasts used as targets were prepared by incubating erythrocyte-depleted spleen cells in culture medium with Con A at 2.5 μg/ml for 3 days at 37°C in 5% CO_2/95% air. Tumor targets were grown in culture medium and used 3 days after the last passage. Targets were labeled by incubation of resuspended cell pellets with 100 μCi of ^{51}Cr (1 Ci = 37 GBq; New England Nuclear) for 1 hr at 37°C and washed four times before use. Effector cells (100 μl) were incubated with 100 μl containing 2×10^4 target cells in 96-well round-bottom culture plates for 4 hr at 37°C in 5% CO_2/95% air. Culture supernatants were harvested with a supernatant collection system (Skatron, Lier, Norway) and radioactivity was determined in a γ counter. Percent specific ^{51}Cr release was calculated by using the formula: [(experimental release) − (spontaneous release)]/[(maximum release) − (spontaneous release)] × 100. Maximum ^{51}Cr release was obtained by incubating target cells with 5% (wt/vol) $NaDodSO_4$. Spontaneous release was obtained by incubating target cells in medium alone. For blocking assays, 50 μl of effector cells was briefly incubated with 50 μl of appropriately diluted mAb prior to the addition of target cells. All mAbs were added as hybridoma culture supernatants enriched for immunoglobulin by ammonium sulfate precipitation and used at a final concentration of 1%. Identification of the antibodies is given above, except for anti-K^b (28-13-3S) and anti-I-A^b (28-16-8S and M5/114.15.2). Percent inhibition was calculated as [(% specific release without antibody) − (% specific release with antibody)]/[(%specific release without antibody)] × 100.

RESULTS

A $CD8^-$ $CD4^-$ T-cell line was established from nylon wool nonadherent C3H/HeJ-*gld*/*gld* lymph node cells by negative selection with anti-CD8, anti-CD4, and anti-Ia mAbs plus complement, followed by allostimulation (16), using C57BL/6 ($H\text{-}2^b$) spleen cells. The line was cloned twice by limiting dilution, generating several Thy-1^+ $CD8^-$ $CD4^-$ clones (16) and one Thy-1^- $CD8^-$ $CD4^-$ clone, termed 2C4.

Surface Phenotype. Analysis of the cell surface phenotype of 2C4 is shown in Fig. 1. The clone was uniformly Thy-1^- $CD8^-$ $CD4^-$ surface immunoglobulin-negative $B220^-$ $H\text{-}2^{k+}$ IL-2 receptor-positive Ly6E.1^+ LFA-1^+ F23.1 (TCR $V_{\beta 8}$)$^-$ $CD3^+$. This phenotype is in contrast to that of freshly isolated *gld* null T-cell populations, which are mostly $B220^+$ and IL-2 receptor-negative (10). The B220 antigen is predominantly a B-cell marker (19) but is induced on certain $CD8^-$ $CD4^-$ thymocytes from normal mice *in vitro* (20). 2C4 did not stain with mAb MEL-14, which recognizes a putative lymph node-homing receptor (21), did not express J11d, a differentiation antigen of unknown function expressed on >90% of mouse thymocytes but not on peripheral T cells (22), and was Ia (major histocompatibility complex class II)-negative (data not shown). Lack of reactivity with the anti-TCR allotypic mAb F23.1 indicated that 2C4 did not express a TCR utilizing a member of the $V_{\beta 8}$ family of TCR variable region genes but did not preclude the expression of other TCR β-chain genes.

RNA Expression. As shown in Fig. 2, 2C4 did not express detectable levels of Thy-1 RNA transcripts, supporting the conclusion that 2C4 lacked Thy-1 proteins on the cell surface and suggesting a pretranscriptional block of Thy-1 gene expression. Equal amounts of RNA were applied to each lane, as judged by ethidium bromide staining of the rRNA species. Southern blot analysis indicated that the Thy-1 gene was present in the clone, on an appropriate size restriction fragment. CD8 and CD4 transcripts were also undetectable.

CD3/TCR Complex Expression. The anti-CD3 ε-chain mAb 2C11 was used to immunoprecipitate ^{125}I-labeled surface proteins from 2C4 lysates to examine the TCR proteins expressed by the clone (Fig. 3 *A* and *B*). Under nonreducing conditions (Fig. 3*A*), anti-CD3 precipitated a complex of 85–95 kDa that was similar to the complex precipitated from an F23.1^+ (α/β TCR^+) cytotoxic T-cell clone (N1D5) generated from normal C3H mice (16). Under reducing conditions (Fig. 3*B*), anti-CD3 precipitated proteins migrating in the range of 35–43 kDa from both 2C4 and N1D5 lysates. In addition, a slightly larger band of ≈50 kDa was observed for the 2C4 but not the N1D5 clone. The identity of this larger species is unknown, but it is probably a more heavily glycosylated TCR β chain, based on nonequilibrium pH gel electrophoresis (data not shown).

It is likely that 2C4 expresses an α/β TCR based on the molecular masses of the proteins precipitated by anti-CD3, their apparent similarity to the proteins precipitated from the α/β TCR-bearing clone N1D5, and the expression by 2C4 of full-length (1.6 kilobases) TCR α and β chains (1.3 kilobases) mRNA (Fig. 3*C*). Expression of a γ/δ TCR is unlikely due to the failure to detect δ-chain RNA transcripts (data not shown). However, since 2C4 also expressed abundant TCR γ-chain RNA, the expression of a γ chain cannot be formally excluded. This pattern of expression is similar to the double-

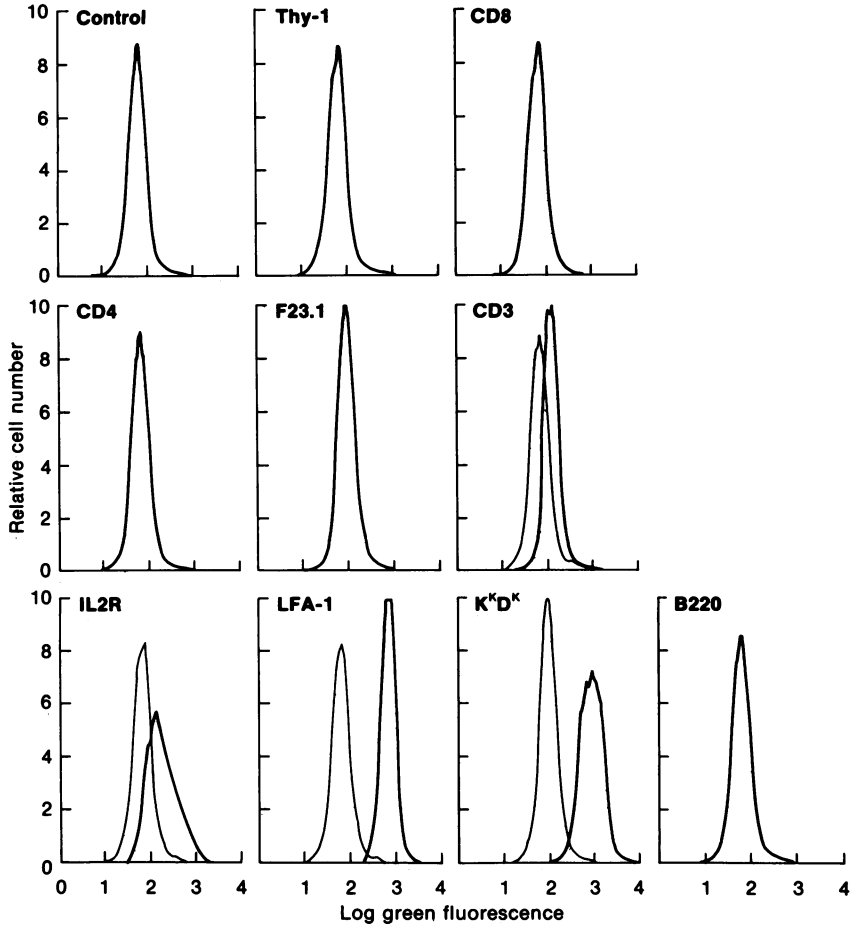

FIG. 1. Flow microfluorimetric analysis of cell surface proteins expressed by clone 2C4. All antibodies exhibited appropriate staining of normal C3H/HeJ spleen or nylon wool nonadherent lymph node cells. The control shows staining with FITC-labeled anti-rat immunoglobulin alone. Identical results were obtained with the FITC-labeled anti-mouse immunoglobulin and FITC-labeled anti-hamster immunoglobulin. IL2R, IL-2 receptor.

negative cytotoxic T-lymphocyte (CTL) clones we have described (16).

Cell Growth. 2C4 required one or more T-cell-derived factors for growth since elimination of supernatant from Con A-activated rat spleen cells from the cultures resulted in complete cell death by 5 days. Periodic stimulation with alloantigen was also required. Growth was IL-2-dependent, since proliferation was completely inhibited by anti-IL-2 receptor antibody. Significant proliferation was observed in response to recombinant IL-2 alone, to Con A in the presence of accessory cells (whole spleen), and to immobilized anti-CD3 in the presence of IL-2.

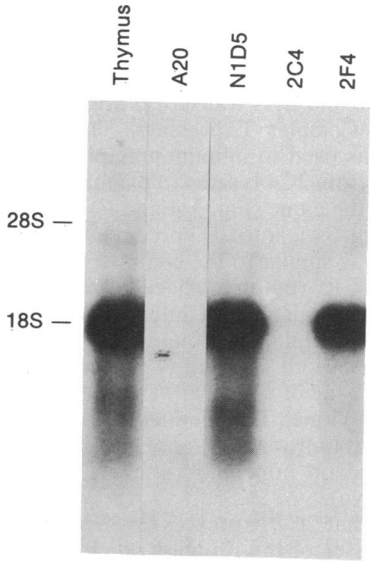

FIG. 2. Northern blot analysis of Thy-1 RNA expression. A ^{32}P-labeled Thy-1 cDNA probe was prepared by primer-extension labeling of the *Pst* I insert of clone pT64 (23), with a specific activity of 5×10^8 cpm/μg. Arrows indicate positions of the 28S and 18S rRNA species. Lanes: thymus, adult C3H/HeJ thymocytes; A20, B-cell lymphoma A20; N1D5, normal C3H lymph node-derived CTL clone (16); 2C4, 2C4 cells; 2F4, Thy-1$^+$ double-negative CTL clone from *gld* lymph node.

Cytotoxic Activity. As shown in Table 1, the Thy-1$^-$ clone exhibited allospecific cytotoxic activity typical of conventional CTLs. No autoreactive CTL activity was detected using coisogenic C3H/HeJ (H-2^k) Con A-blasts as targets. Titratable lytic activity was observed against congenic (H-2^b) C3H.SW and C57BL/6 (H-2^b) Con A-blasts, and the H-2^b thymoma EL4. Lysis of C3H.SW suggested the response mapped to the H-2 locus. Killing of B10.A (5R) (K^bD^d) but not B10.A (2R) (K^kD^b) suggested the killing was K^b-specific. These data were supported by the almost complete inhibition of lysis by anti-K^b mAb and the lack of inhibition by anti-D^b or anti-Ia^b (Table 2 and data not shown).

Fine specificity was analyzed against a panel of target cells containing known mutations in the K^b molecule (K^{bm} mutants). Of the available mutants, significant lysis was observed only with *bm7* target cells, comparable to lysis of wild-type B6 target cells, a positive control in the same assay. The K^{bm7} mutant contains amino acid substitutions at positions 116 and 121, both in the external α-chain domain 2 (27). By analogy with the three-dimensional structure of the human class I HLA-A2 molecule (28), the *bm7* mutant is the only target whose mutations lie solely within the presumed antigen-binding groove (in the β-sheet forming the floor of the groove), an area not thought to interact directly with the TCR (29). The other mutants contain known mutations in the α-chain domain 1 (*bm3*, *-8*, and *-11*) or α-chain domain 2 (*bm1*

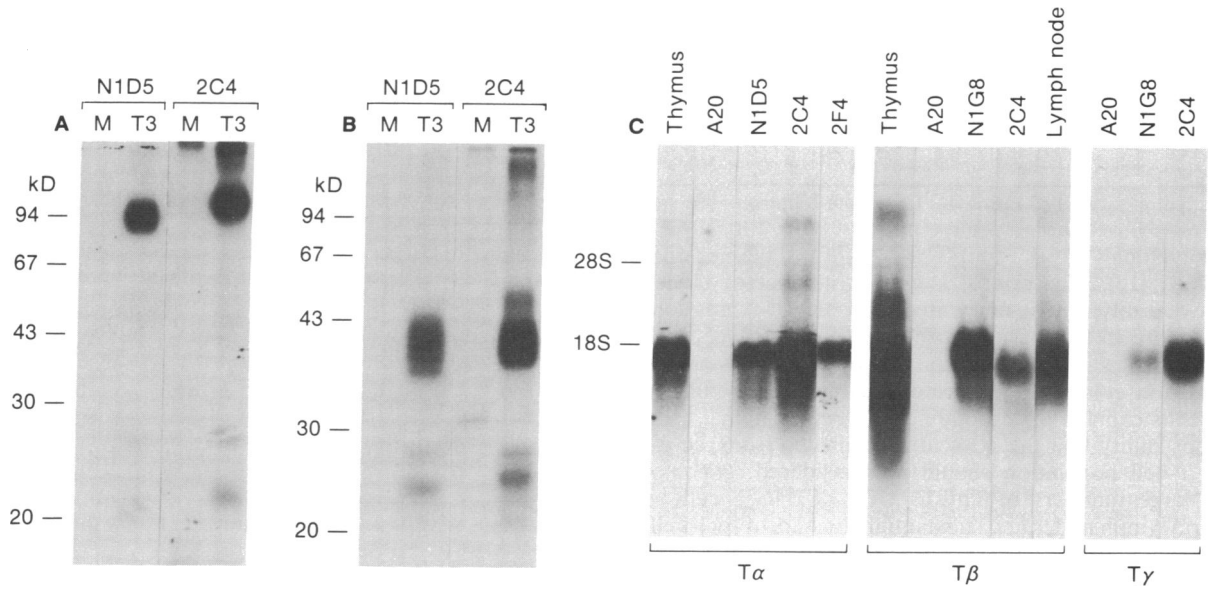

FIG. 3. Gel electrophoresis of ^{125}I-labeled surface proteins immunoprecipitated with anti-CD3 (2C11; lanes T3) under nonreducing (*A*) or reducing (*B*) conditions. Cell lysates were prepared in 1% digitonin buffer. Lanes M, medium only. N1D5 is a CD8$^+$ F23.1$^+$ CTL clone derived from normal C3H lymph node (16). (*C*) Northern blot analysis of TCR α-, β-, and γ-chain RNA. Hybridizations were carried out with a ^{32}P-labeled Tα probe (*Nco* I fragment of pHDS58, ref. 24), Tβ cDNA probe (*Eco*RI fragment of pUC 25, ref. 25), or Tγ cDNA probe (*Pvu* II fragment of pHDS205, ref. 26). Arrows indicate positions of the 28S and 18S rRNA species. Lanes: thymus, normal adult C3H thymocytes; A20, B-cell lymphoma A20; N1D5 and N1G8, α/β TCR$^+$ CTL clones derived from normal C3H lymph node; lymph node, normal C3H nylon wool nonadherent lymph node cells; 2C4, 2C4 cells; 2F4, Thy-1$^+$ double-negative clone from C3H-*gld*/*gld* lymph node.

and -*10*) (27). The data show that, similar to anti-K^b CTLs from normal mice (27), amino acid residues in both external domains of the K^b molecule are important for target recognition by 2C4. The fact that mutations in the K^b molecule can abolish lytic activity is further evidence of K^b specificity.

2C4 did not exhibit significant lysis of third party (H-2^s, H-2^d) targets and did not kill the murine natural killer cell-susceptible target YAC-1. The clone is, therefore, functionally distinct from conventional natural killer cells and from γ/δ TCR$^+$ double-negative T cells that exhibit major histocompatibility complex nonspecific cytolytic activity (18, 30).

As shown in Table 2, anti-Thy-1, anti-CD8, anti-CD4, anti-I-A^b, and anti-TCR F23.1 all failed to inhibit killing by 2C4. Lysis was almost completely abrogated by the presence of anti-K^b, anti-CD3 (2C11), or anti-LFA-1 in the assay, at concentrations as low as 0.1% of the assay volume. Inhibition by anti-CD3 suggests that the CD3/TCR complex expressed by 2C4 (presumably an α/β heterodimer) was involved in recognition and cytolysis. Also, immobilized anti-CD3 induced significant proliferation of the clone, further suggesting that the CD3/TCR complex was functional (data not shown).

Table 1. Specificity of cell killing by 2C4

Target cell	% specific release of ^{51}Cr			
	10:1	5:1	2.5:1	1.3:1
Con A-blast				
C57BL/6 (H-2^b)	25.2	15.0	3.0	<0
C3H/HeJ (H-2^k)	<0	<0	<0	<0
C3H.SW (H-2^b)	55.4	45.6	33.2	23.7
B10.A (2R) (K^{k}D^b)	2.0	<0	<0	<0
B10.A (5R) (K^{b}D^d)	36.0	16.0	17.0	8.0
bm1	8.8	3.2	4.7	1.2
bm3	0.9	<0	<0	2.2
bm7	31.7	20.8	13.1	11.5
bm8	3.0	<0	<0	0.7
bm10	8.7	5.6	1.2	5.5
bm11	4.4	<0	0.3	<0
A.SW (H-2^s)	<0	0.9	2.1	0.9
BALB/c (H-2^d)	<0	<0	<0	<0
Tumor cell				
YAC-1 (H-2^a)	<0	<0	<0	<0
EL4 (H-2^b)	59.7	53.3	47.2	38.1
A20 (H-2^d)	4.9	2.6	2.7	1.6
P815 (H-2^d)	<0	<0	4.1	1.0
K562 (human)	0.8	1.9	1.8	0.5

Effector/target ratio of 10:1, 5:1, 2.5:1, and 1.3:1 were used.

DISCUSSION

The major conclusion that can be drawn from these results is that, at least in cells derived from *gld* mice, surface expression of Thy-1 (and CD8 or CD4) is not required for recognition or killing by allospecific CTLs and that Thy-1 expression is not obligatory for T-cell growth or expression of CD3/α/β TCR complexes on the cell surface. The data also raise the possibility that Thy-1 expression may not be obligatory during the ontogeny of major histocompatibility complex specific CTLs in *gld* mice.

The clone we describe lacked expression of Thy-1 and the T-cell accessory molecules CD8 and CD4 at the level of gene transcription. Southern blotting revealed that the Thy-1 gene was present and showed no evidence of any gross deletion or rearrangement of the gene. It is, of course, still possible that a spontaneous or induced mutation prevented expression of Thy-1 and the clone is, therefore, not representative of a Thy-1$^-$ T-cell type naturally present in *gld* mice. The clone expressed Ly6E.1 on the cell surface, arguing that lack of Thy-1 expression was not due to some feedback mechanism resulting from a general defect in the addition of glycolipid membrane anchors to surface proteins. Whether due to mutation or not, lack of Thy-1 expression clearly did not prevent expression of functional CD3/TCR complexes, cell growth, or mediation of allospecific CTL activity. In most of the assays reported, Thy-1$^-$ and Thy-1$^+$ double-negative clones were analyzed simultaneously, and their responses were indistinguishable. This is in contrast to earlier studies that clearly showed functional differences between noncul-

Table 2. Inhibition of 2C4 killing by mAbs

Target cell	E/T	% ^{51}Cr release (control)	% inhibition of killing							
			Anti-Thy-1	Anti-CD8	Anti-CD4	Anti-CD3	Anti-LFA-1	Anti-TCR	Anti-I-A^b	Anti-K^b
C57BL/6	6:1	44	2	0	0	83	92	0	0	ND
EL4	8:1	66	0	0	0	94	94	0	0	88

E/T, effector/target cell ratio. % ^{51}Cr release (control) = % specific release of ^{51}Cr with no mAb added. ND = not done.

tured polyclonal Thy-1$^-$ and Thy-1$^+$ *gld* double-negative populations (15). Analysis of a greater number of clones might reveal differences correlated with Thy-1 expression. Perhaps more likely, based on the low responses of fresh populations to *in vitro* stimulation, only certain cells can be propagated in culture and, therefore, clones may be biased toward cells capable of responding to T-cell stimuli.

The possibility that the 2C4 clone originated from the *gld* Thy-1$^-$ T-cell population should be considered. *gld* mice contain large numbers of Thy-1$^-$ CD8$^-$ CD4$^-$ T cells in peripheral lymph nodes and substantial numbers of such cells were present in the CD8$^-$ CD4$^-$ cell line from which 2C4 was derived. Although freshly isolated *gld* null T-cell populations give minimal specific responses to alloantigen, they proliferate in response to IL-2 or supernatant from Con A-activated rat spleen cells. These responses may be enhanced in the presence of syngeneic or allogeneic spleen cells (S.W., K.Y., and M.I.G., unpublished). Therefore, it is possible that our original cultures allowed a short-term non-antigen-specific expansion of Thy-1$^-$ T cells and subsequent selection of antigen-specific cells such as 2C4.

gld Thy-1$^-$ cells may develop without ever expressing Thy-1. Alternatively, all *gld* double-negative T cells express Thy-1 at some point in their ontogeny and a large proportion (≈20%) subsequently stop transcribing the gene. None of our Thy-1$^+$ double-negative clones have lost Thy-1 in culture. If the 2C4 clone represents a Thy-1$^-$ T cell naturally present in *gld* mice, then whatever the timing of prevention/loss of Thy-1 expression *in vivo*, it is not sufficient to prevent subsequent differentiation to the CTL effector stage in at least some cases.

We have suggested (16) several models for the *in vivo* origins of the Thy-1$^-$ and Thy-1$^+$ double-negative T cells in the periphery of *gld* mice. One scenario is that they represent normal cells at a particular stage of differentiation or activation that are greatly expanded due to the effect(s) of the *gld* mutation. Thy-1$^-$ α/β TCR$^+$ double-negative thymocytes and Thy-1$^+$ CD8$^-$ CD4$^-$ α/β TCR$^+$ thymocytes and peripheral T cells have been detected in normal mice (20, 31, 32). Similar cells are also present in peripheral blood of sheep (33). Some of these cells express the B220 antigen, similar to *gld* double negatives. The functional capacities of these populations and their relationship to other T-cell lineages are not known. It is interesting to speculate that such normal α/β TCR$^+$ double negatives may be the source of the expanded populations in the *gld* mouse periphery. A similar scenario has been suggested in the case of mice bearing the nonallelic mutation *lpr* (20). *lpr* mice also accumulate large numbers of peripheral double-negative T cells (12).

It will be of interest to determine whether 2C4 can be induced to express T-cell differentiation antigens *in vitro* or *in vivo* and whether Thy-1$^-$ CTLs can be generated from normal mice. Also, this clone may be of interest for future studies using gene transfection to investigate the role(s) of Thy-1 and CD8 in CTL differentiation and function.

We thank Dr. Allan Pickard for the fluorescence-activated cell sorter analyses, Drs. John E. Coligan, Fritz Koning, and Yasuo Kokai for advice, Dr. Jeffrey Bluestone for the 2C11 antibody, Dr. David Raulet for a TCR δ probe, and Drs. Glen Gaulton and Yasuhiro Hashimoto for critical reviews. This work was supported by grants from the National Institutes of Health, National Cancer Institute, and National Eye Institute.

1. Romani, N., Stingl, G., Tschachler, E., Witmer, M. D., Steinman, R. M., Shevach, E. M. & Schuler, G. (1985) *J. Exp. Med.* **161**, 1368–1383.
2. Reif, A. E. & Allen, J. M. (1966) *Nature (London)* **209**, 523.
3. Palacios, R., Kiefer, M., Brockhaus, M., Karjalainen, K., Dembic, Z., Kisielow, P. & von Boehmer, H. (1987) *J. Exp. Med.* **166**, 12–32.
4. Ceredig, R., Dialynas, D. P., Fitch, F. W. & MacDonald, H. R. (1983) *J. Exp. Med.* **158**, 1654–1671.
5. Gunter, K. C., Malek, T. R. & Shevach, E. M. (1984) *J. Exp. Med.* **159**, 716–730.
6. Tentori, L., Pardoll, D. M., Zuniga, J. C., Hu-Li, J., Paul, W. E., Bluestone, J. A. & Kruisbeek, A. M. (1988) *J. Immunol.* **140**, 1089–1094.
7. Kroczek, R. A., Gunter, K. C., Seligmann, B. & Shevach, E. M. (1986) *J. Immunol.* **136**, 4379–4384.
8. Kroczek, R. A., Gunter, K. C., Germain, R. N. & Shevach, E. M. (1986) *Nature (London)* **322**, 181–184.
9. Gunter, K. C., Germain, R. N., Kroczek, R. A., Saito, T., Yokoyama, W. M., Chan, C., Weiss, A. & Shevach, E. M. (1987) *Nature (London)* **326**, 505–507.
10. Roths, J. B., Murphy, E. D. & Eicher, E. M. (1984) *J. Exp. Med.* **159**, 1–20.
11. Davidson, W. F., Holmes, K. L., Roths, J. B. & Morse, H. C., III (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1219–1223.
12. Davidson, W. F., Dumont, F. J., Bedigian, H. G., Fowlkes, B. J. & Morse, H. C., III (1986) *J. Immunol.* **136**, 4075–4084.
13. Hashimoto, Y., Yui, K., Littman, D. & Greene, M. I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5883–5887.
14. Samelson, L. E., Davidson, W. F., Morse, H. C., III, & Klausner, R. D. (1986) *Nature (London)* **324**, 674–676.
15. Yui, K., Hashimoto, Y. & Greene, M. I. (1988) *Immunol. Res.* **7**, 173–188.
16. Yui, K., Wadsworth, S., Yellen, A., Hashimoto, Y., Kokai, Y. & Greene, M. I. (1988) *Immunol. Rev.* **104**, 121–155.
17. Iwamoto, A., Rupp, F., Ohashi, P. S., Walker, C. L., Hanspeter, P., Joho, R., Hengartner, H. & Mak, T. W. (1986) *J. Exp. Med.* **163**, 1203–1212.
18. Lew, A. M., Pardoll, D. M., Maloy, W. L., Fowlkes, B. J., Kruisbeek, A., Cheng, S.-F., Germain, R. N., Bluestone, J. A., Schwartz, R. H. & Coligan, J. E. (1986) *Science* **234**, 1401–1405.
19. Scheid, M. P., Landreth, K. S., Tung, J.-S. & Kincade, P. W. (1982) *Immunol. Rev.* **69**, 141–159.
20. Fowlkes, B. J., Kruisbeek, A. M., Ton-That, H., Weston, M. A., Coligan, J. E., Schwartz, R. H. & Pardoll, D. M. (1987) *Nature (London)* **329**, 251–254.
21. Gallatin, W. M., Weissman, I. L. & Butcher, E. C. (1983) *Nature (London)* **304**, 30–34.
22. Bruce, J., Symington, F. W., McKearn, T. J. & Sprent, J. (1981) *J. Immunol.* **127**, 2496–2501.
23. Moriuchi, T., Chang, H.-C., Denome, R. & Silver, J. (1983) *Nature (London)* **301**, 80–82.
24. Saito, H., Kranz, D. M., Takagaki, Y., Hayday, A. C., Eisen, H. N. & Tonegawa, S. (1984) *Nature (London)* **312**, 36–40.
25. Bier, E., Hashimoto, Y., Greene, M. I. & Maxam, A. M. (1985) *Science* **229**, 528–534.
26. Saito, H., Kranz, D. M., Takagaki, Y., Hayday, A. C., Eisen, H. N. & Tonegawa, S. (1984) *Nature (London)* **309**, 757–762.
27. Nathenson, S. G., Geliebter, J., Pfaffenbach, G. M. & Zeff, R. A. (1986) *Annu. Rev. Immunol.* **4**, 471–502.
28. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329**, 506–512.
29. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329**, 512–518.
30. Bluestone, J. A., Pardoll, D., Sharrow, S. O. & Fowlkes, B. J. (1987) *Nature (London)* **326**, 82–84.
31. Scollay, R., Wilson, A., D'Amico, A., Kelly, K., Egerton, M., Pearse, M., Wu, L. & Shortman, K. (1988) *Immunol. Rev.* **104**, 81–120.
32. Budd, R. C., Miescher, G. C., Howe, R. C., Lees, R. K., Bron, C. & MacDonald, H. R. (1987) *J. Exp. Med.* **166**, 577– 582.
33. Mackay, C. R., Maddox, J. F. & Brandon, M. R. (1987) *Cell. Immunol.* **110**, 46–55.