

CHARACTERIZATION OF Lyt-2⁻,L3T4⁻ CLASS I-SPECIFIC CYTOLYTIC CLONES IN C3H-*gld/gld* MICE

Implications for Functions of Accessory Molecules and Programmed Development

BY KATSUYUKI YUI, YASUHIRO HASHIMOTO, SCOTT WADSWORTH, AND
MARK I. GREENE

*From the Division of Immunology, Department of Pathology and Laboratory Medicine, School of
Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

The Lyt-2 and L3T4 molecules are expressed on two nonoverlapping populations of normal murine peripheral T cells, namely, the Lyt-2⁺,L3T4⁻ and the Lyt-2⁻,L3T4⁺ subsets (1-6). The phenotype of mature T cells generally correlates with their function. Helper T cells are predominantly Lyt-2⁻,L3T4⁺, whereas most T killer cells and their precursors belong to the Lyt-2⁺,L3T4⁻ subset (7-9). Expression of these molecules also correlates with MHC recognition patterns (1-5, 10, 11). In general, Lyt-2⁺,L3T4⁻ T cells recognize class I structures and Lyt-2⁻,L3T4⁺ T cells recognize class II molecules, although exceptions have been reported (12-14). In addition, antibodies directed against the Lyt-2 or L3T4 structures appear to inhibit some functional properties of each respective T cell subset in vitro (1, 4, 10). It has been proposed that Lyt-2 and L3T4 participate in T cell antigen recognition by binding to some non-polymorphic portion of the MHC molecules. These structures are assumed to stabilize the TCR and increase the overall avidity of the TCR for its target molecules (15, 16), although no direct evidence exists to support this hypothesis.

T cell accessory molecules may also be involved in transmembrane signaling that regulates the activation process of T cells (17, 18). This notion is based on the observation that anti-L3T4 mAbs inhibit Con A- or anti-T3 mAb-induced T cell activation in assay systems free of MHC class II antigen-bearing cells. Little biochemical information exists to explain these observations.

In contrast to the major subsets of T cells, a minor population of CD4⁻,CD8⁻ T cells has been identified in thymus and peripheral organs. The thymic double negative (CD4⁻,CD8⁻) cell subset may contain precursor T cells (19, 20). In addition, cell surface γ chain polypeptides have been identified in some thymic and peripheral double-negative cells (21, 22), indicating that this protein is expressed independently of accessory molecules.

The mutant strain of C3H/HeJ, C3H/HeJ-*gld/gld*, develops a lupus-like au-

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¹ Abbreviation used in this paper: CTLp, CTL precursor.

toimmune syndrome and age-related massive lymph node enlargement (23). Analysis of C3H-*gld/gld* lymph node residing lymphoid subsets by immunofluorescence indicates an increase in the numbers of B cells, T cells, and null cells (Thy-1⁻, surface Ig⁻) compared with cells found in C3H-+/+ mice. In addition, most lymph node residing T cells are of the Lyt-2⁻,L3T4⁻ subset.

We have been involved in the study of T cell receptor gene and cell surface protein expression of these lymph node accumulating T cells in C3H-*gld/gld* (24, 25; Hashimoto, Y., K. Yui, J. Krawiec, and M. I. Greene, manuscript submitted for publication; Yui, K., Y. Hashimoto, and M. I. Greene, manuscript submitted for publication). The analysis revealed that Thy-1⁺,Lyt-2⁻,L3T4⁻ cells and Null cells expressed TCR proteins on their cell surface. Functional analysis of the double-negative cells showed that they proliferate after stimulation with T cell mitogens such as Con A or alloantigen in the presence of an exogenous source of rat Con A supernatant. These responses were, however, reduced when compared with the response pattern of normal peripheral T cells.

In this report, we describe Thy-1⁺,Lyt-2⁻,L3T4⁻ alloreactive cytotoxic T cell clones derived from the double-negative cell subset found in C3H-*gld/gld* lymph nodes. Proliferation of these double-negative cell lines is IL-2 dependent. The clones lysed target cells that express H-2K^d molecules even though the effector clones lack both Lyt-2 and L3T4 structures. Collectively, these results indicate that both Lyt-2 and L3T4 structures are not essential for induction, recognition, and killing of alloantigen-specific CTL. We discuss the expression and function of these TCR accessory structures in the development and function of T cells.

Materials and Methods

Mice. C3H/HeJ-*gld/gld* mice were obtained from The Jackson Laboratory (Bar Harbor, ME), bred in our colonies at the University of Pennsylvania, and were used after the age of 3 mo. C3H/HeJ-+/+, BALB/cJ, B10.BR, B10.D2, B10.A, and B10 mice were purchased from The Jackson Laboratory and were used when 6–12 wk of age.

Antibodies. The hybridoma cell lines F23.1.2R (anti-TCR allotype) (26) and 3C7 (anti-IL-2-R) (27) were generous gifts from Dr. Michael Bevan (Scripps Clinic and Research Foundation, La Jolla, CA) and Dr. Ethan M. Shevach (National Institute of Allergy and Infectious Diseases, Bethesda, MD), respectively. 30-H12 (anti-Thy-1) (28), 3.155 (anti-Lyt-2) (29), 116-13.4 (anti-Lyt-2) (30), GK1.5 (anti-L3T4) (4), 34-1.2S (anti-H-2K^d) (31), 28-16-8S (anti-I-A^{b,d}) (32), and 14-4-4S (anti-I-E) (33) were obtained from American Type Culture Collection (Rockville, MD). The Ig from culture supernatants of these hybridomas was enriched by ammonium sulfate precipitation. Culture supernatant of KJ16-133 (34) was a gift of Drs. John Kappler and Philippa Marrack (National Jewish Hospital and Research Center, Denver, CO).

Interleukin 2. Con A supernatant was prepared from Fisher-344 rat spleen cells and supplemented with 0.2 M α -methyl-D-mannoside to neutralize the remaining Con A. Con A supernatant was used at 5 or 10% final concentration in culture. Human rIL-2 was obtained from Cetus Corp. (Emeryville, CA).

Establishment of Double-negative Cell Lines and Clones. Cell cultures were carried out in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 M nonessential amino acids, 5×10^{-5} M 2-ME, penicillin/streptomycin, and 10% FCS. C3H-*gld/gld* double-negative cells were prepared as described elsewhere (Yui, K., Y. Hashimoto, and M. I. Greene, manuscript submitted for publication). Briefly, the nylon-wool nonadherent population of C3H-*gld/gld* lymph node lymphocytes was treated with anti-Lyt-2, anti-L3T4, and anti-I-E mAbs and complement (Pel-Freez Biologicals, Rogers, AR). Viable cells were recovered by centrifugation on Ficoll-Paque (Pharmacia Fine

Chemicals, Piscataway, NJ). 2×10^6 cells/ml double-negative cells were cultured with 2×10^6 /ml irradiated (2,000 rad) BALB/c spleen cells in the presence of 5% rat Con A supernatant. A bulk in vitro double-negative cell line (LL line) was established by weekly alloantigenic stimulation followed by rest periods using C3H-*gld/gld* double-negative cells with BALB/c or C3H-+/+ irradiated spleen cells in medium supplemented with 5% rat Con A supernatant. In the first of several stimulations, proliferation of the cell line was poor, probably reflecting the limited frequency of proliferating cells. After stable growth of the cell line was obtained, the LL line was stimulated weekly by BALB/c spleen cells without intervening rest periods. The LL line was maintained by plating at 5×10^4 /ml with 2×10^6 /ml irradiated BALB/c spleen cells and 5% rat Con A supernatant.

LL cells were next cloned by limiting dilution. 24 clones grew in two plates distributed at final concentrations of 0.3 cells/well (cloning efficiency 30%). The expression of TCR allotypic antigens (KJ16 and F23) on these clones was tested using antibody-mediated cytotoxicity. Clones positive for KJ16 or F23 were expanded for further analysis. We further selected clones that grew very well and finally obtained three double-negative clones. Clones were routinely passaged weekly with 5×10^4 /ml cloned lymphocytes in Con A supernatant-supplemented medium and were stimulated with 2×10^6 irradiated BALB/c spleen cells per milliliter. Normal C3H/HeJ clones N1G8 and N1D5 were obtained from lymph node cells by limiting dilution and stimulated with irradiated BALB/c spleen cells in the presence of rat Con A supernatant.

Flow Cytometric Analysis. The LL line and clones were harvested from culture and nonviable stimulator cells were separated by Ficoll-Paque before staining. C3H-+/+ splenic T cells were prepared from C3H-+/+ nylon-wool nonadherent spleen cells. For fluorescent staining, 10^6 cells were incubated in 100 μ l PBS (0.01 M, pH 7.2) supplemented with 0.5% BSA and 0.1% sodium azide with various mAbs for 20 min at 4°C. After washing once, the pellets were incubated with 50 μ l FITC-coupled anti-rat κ chain mAb (Becton Dickinson & Co., Mountain View, CA) at 4°C for an additional 20 min. After three washes, the cells were analyzed on a FACS IV (Becton Dickinson & Co.).

Proliferation Assay. 200 μ l of 10% FCS-supplemented medium containing $1-2 \times 10^4$ responder cells and 2×10^5 irradiated (2,000 rad) spleen cells was placed in each well of a sterile U-bottomed microculture plate and incubated at 37°C in a humidified atmosphere of 5% CO₂. After 4 d of culture, proliferative responses were assessed by 16-h exposure to 1 μ Ci [³H]thymidine. rIL-2, rat Con A supernatant, and mAbs were added in culture when indicated. The data were expressed as counts per minute (mean \pm SEM).

CTL Assay. LL cells or clones were used in CTL assays 4-6 d after stimulation. Cells (100 μ l) were mixed with $1-2 \times 10^4$ ⁵¹Cr-labeled targets (100 μ l) in round-bottomed microtiter plates. After incubation at 37°C for 4 h, supernatants were harvested by a supernatant collection system (Skatron AS, Lier, Norway) and counted for radioactivity. Percent specific lysis was calculated as: percent specific lysis = $100 \times [(\text{experimental release}) - (\text{spontaneous release})] / [(\text{maximal release}) - (\text{spontaneous release})]$.

Spontaneous release was measured by incubating target cells without CTL, and maximal release was measured by incubating target cells in 5% SDS. For the blocking study, 50 μ l of appropriately diluted antibody preparations were placed in microtiter wells with 100 μ l of target cells and 50 μ l of CTLs.

As target cells, Con A blasts were prepared by culturing spleen cells in the presence of 2 μ g/ml Con A for 3 d. Cells were labeled by incubating at 37°C in the presence of ⁵¹Cr for 1 h, and were washed before use.

Northern Blots. Lyt-2 (35), L3T4 (36), C α (pHDS58) (37), C γ (pHDS205) (38), and T3 δ (pPGBC-9) (39) probes were gifts of Drs. Jane R. Parnes (Stanford University, Stanford, CA), Dan R. Littman (University of California, San Francisco, CA), Susumu Tonegawa (MIT, Boston, MA), and Cox Terhorst (Dana-Farber Cancer Institute, Boston, MA), respectively. Inserts of pHDS58, pUC25 (40), and pHDS205 were labeled by the oligo-labeling method (41). Lyt-2, L3T4, and T3 δ probes were labeled by nick translation (42).

Total cellular RNA was extracted from nylon-wool nonadherent C3H/HeJ lymph node cells, C3H/HeJ thymocytes, and various clones. Total RNA was denatured with formal-

dehyde, subjected to electrophoresis through a 1% agarose gel, and transferred to Genescreen plus (DuPont Co., Wilmington, DE). After prehybridization, the membrane was hybridized with the labeled probe in 50% formamide, 3× SSC (20× SSC: 3 M sodium chloride and 0.2 M sodium citrate), 5× Denhardt's solution, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20 µg/ml sheared and denatured salmon sperm DNA, 0.75% SDS and 10% dextran sulfate for 20 h at 42°C. After hybridization, filters were washed twice in 2× SSC at room temperature and twice in 2× SSC/1.0% SDS for 30 min at 65°C. Washed filters were exposed to x-ray film at -70°C in the presence of intensifying screens.

Results

Establishment of a Bulk-cultured Double-negative Cell Line and Clones. Lyt-2⁻,L3T4⁻ cells residing in the lymph nodes of C3H-*gld/gld* mice were obtained by treating lymph node cells with anti-Lyt-2, anti-L3T4, and anti-Ia mAb and complement. A long-term cultured alloreactive double-negative cell line (we refer to this as the LL line) was established by repeated stimulation of C3H-*gld/gld* double-negative cells with irradiated BALB/c spleen cells in the presence of 5% rat Con A supernatant. The line was cloned by limiting dilution. Only F23⁺ clones were selected for further study.

Several months after repeated stimulation, the cell surface phenotype of the cell line and clones was analyzed (Fig. 1). In contrast to the C3H-+/+ nylon-wool column-passed lymph node cells or the C3H-+/+-derived Lyt-2⁺ clone N1D5, the surface phenotype of the LL line was Thy-1⁺,Lyt-2⁻,L3T4⁻. Three clones obtained from this line maintained the original phenotype. The fluorescence profile of one of these double-negative clones, L3C7 (Thy-1⁺,Lyt-2⁻,L3T4⁻,KJ16⁺,F23⁺) is shown in Fig. 1.

Although these double-negative cells did not express determinants that bound to anti-Lyt-2 or L3T4 mAbs, it was possible that these cells expressed abnormal accessory structures that lacked the determinant reactive with the mAbs used. To address this issue and also to study whether expression of Lyt-2 and L3T4 molecules is regulated at the transcriptional or posttranscriptional levels, we purified total RNA from double-negative clones and analyzed the expression of relevant RNA species by Northern blotting (Fig. 2). Northern blots were hybridized with ³²P-labeled Lyt-2 or L3T4 cDNA probes. C3H/HeJ lymph node T cells and thymocytes were used as positive controls both for Lyt-2 and L3T4 RNA expression. Clones N1G8 and N1D5, derived from normal C3H/HeJ mice and expressing Lyt-2 molecules on the cell surface are positive controls for Lyt-2 RNA. Neither Lyt-2 nor L3T4 mRNA was detectable in the total RNA of double-negative clones.

Factors Required for Growth of the Double-negative Cell Line. We have previously observed (Yui, K., Y. Hashimoto, and M. I. Greene, manuscript submitted for publication) that rat Con A supernatant, but not rIL-2 by itself, is able to sustain the primary MLR of double-negative cells. The factor(s) necessary for the growth of the LL line was studied (Table I). No proliferative response was observed when IL-2 was omitted from culture. rIL-2 sustained the proliferative response of the LL line. As expected, rat Con A supernatant also sustained the MLR response and anti-IL-2 receptor antibody 3C7 completely blocked growth of the LL line in the presence of Con A sup. These results demonstrate that IL-2 is sufficient for the growth and MLR response of the LL line during brief culture

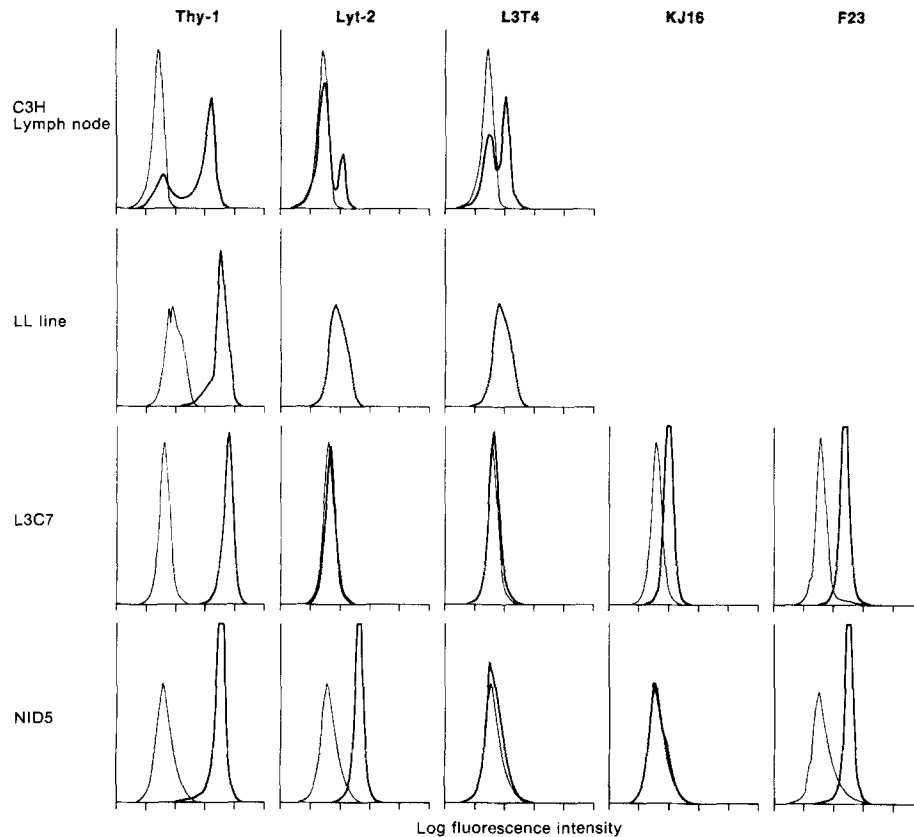


FIGURE 1. Flow cytometric analysis of LL line cell surface markers. C3H-+/+ splenic T cells were a nylon-wool column nonadherent population of C3H-+/+ splenic lymphocytes. The LL line, double-negative clone L3C7 and C3H-+/+-derived clone N1D5 were harvested from culture several days after BALB/c stimulation in the presence of 5% rat Con A supernatant. Cells were stained with anti-Thy-1 (30-H.12), anti-Lyt-2 (3.155), anti-L3T4 (GK 1.5), anti-TCR KJ16 and F23 mAbs and FITC-conjugated anti-rat κ chain mAb or FITC-conjugated goat anti-mouse Fab antibody. The control shows the FACS profile of FITC anti-rat- κ chain mAb alone for Thy-1, Lyt-2, L3T4, KJ16, and FITC anti-mouse Fab alone for F23 antibody.

periods. This contrasts with the requirement of additional factors for the proliferation of bulk double-negative cells in a primary MLR. Collectively, these data suggest that the cocktail of factors that are necessary for the primary response of bulk double-negative cells is distinct from that required for activated clones. Alternatively, distinct subsets of double-negative T cells may have different cytokine requirements and the LL line represents cells that can be sustained by IL-2 alone.

Specificity of the MLR Response of the LL Line. Since Lyt-2 and L3T4 molecules have been associated with the restriction pattern of T cells to class I and class II molecules, respectively (2-6, 10, 11), it was of interest to evaluate whether the LL line recognizes class I or class II molecules. The specificity of the LL line was investigated by blocking proliferative responses against BALB/c stimulators in the presence of rat Con A supernatant (Table II). The proliferative responses of the LL line to C3H spleen cells are background level responses and are not due

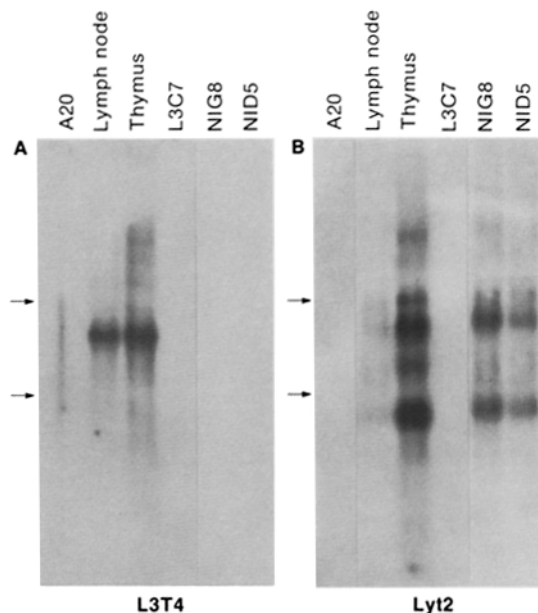


FIGURE 2. Northern blot analysis of Lyt-2 and L3T4 RNA from a double-negative clone. 10 μ g of total RNA prepared from mouse tissue or cell lines was separated on a 1.0% agarose gel, blotted onto Genescreen plus and hybridized with 32 P-labeled Lyt-2 or L3T4 cDNA probes. The positions of the 28S and 18S rRNA markers are indicated. A20, B cell lymphoma A20; lymph node, C3H-+/+ nylon-wool column-passed lymph node cells; thymus, C3H-+/+ thymocytes; L3C7, double-negative clone L3C7; N1G8, Lyt-2⁺ clone N1G8; N1D5, Lyt-2⁺ clone N1D5.

TABLE I
IL-2 Dependency of Growth of LL Lines

Factor added*	mAb added [‡]	[³ H]TdR incorporation [§]			
		Anti-C3H	Anti-BALB/c	Δ cpm	Percent inhibition
—	—	672 \pm 105	664 \pm 293	<0	—
rIL-2 (1.25 U)	—	1,265 \pm 330	5,294 \pm 4	4,029	—
rIL-2 (2.50 U)	—	3,432 \pm 332	10,805 \pm 401	7,373	—
Con A Sup	—	1,328 \pm 253	8,648 \pm 588	7,320	—
Con A Sup	3C7 (3%)	158 \pm 7	329 \pm 141	234	97
Con A Sup	3C7 (1%)	204 \pm 34	600 \pm 41	396	95

* rIL-2 or 5% rat Con A supernatant was added in culture.

[‡] 3C7 is rat anti-mouse IL-2-R antibody that blocks the binding of IL-2 to IL-2-R.

[§] 2×10^4 LL line cells were cultured with 2×10^5 irradiated (2,000 rad) C3H-+/+ spleen cells.

to contaminating autoreactive clones. The basal proliferative response of the LL line alone was similar to these background anti-C3H responses. Basal proliferation was also apparent when cloned double-negative cells were studied.

Various mAbs directed to cell surface structures present on either LL cells or on BALB/c stimulator cells were added to the cultures. As expected from the phenotype, the proliferative response of the LL cells to either C3H or BALB/c stimulators was not significantly inhibited by either anti-L3T4 or anti-Lyt-2 antibodies. The mAb 34-1.2, which reacts with both H-2K^d and D^d molecules, inhibited the proliferative response of the LL line to BALB/c (H-2 K^dD^d) by 82%. In contrast, neither anti-I-A^d nor anti-I-E antibody, both of which react with class II molecules on stimulator cells, had any significant inhibitory effects. We tested various concentrations of these mAbs and similar blocking effects were observed even at a final concentration of 0.1%. In addition, another mAb

TABLE II
Effect of mAbs on the MLR Response of the LL Line

mAb added	Specificity of mAb	Proliferative response of LL line against stimulator cells			
		Anti-C3H	Anti-BALB/c	Δ cpm	Percent inhibition
—	—	12,310 ± 1,155	35,592 ± 2,914	23,282	—
GK1.5	L3T4	9,770 ± 304	31,583 ± 473	21,813	6
116-13.4	Lyt-2	8,666 ± 1,282	27,807 ± 230	19,141	18
34-1.2	K ^d D ^d	8,792 ± 657	13,001 ± 1,346	4,205	82
28-6-8S	I-A ^d	9,310 ± 1,113	32,318 ± 3,083	23,008	1
14-4-4	I-E	8,365 ± 44	29,079 ± 1,548	20,714	11
3C7	IL-2-R	594 ± 293	581 ± 130	0	100

10⁴ LL line cells were cultured with 2 × 10⁵ C3H-+/+ or BALB/c irradiated (2,000 rad) spleen cells in the presence of 10% rat Con A supernatant. mAbs were added at a final concentration of 1%. After 4 d of culture, proliferative responses were assayed by 24-h exposure to 1 μCi of [³H]TdR.

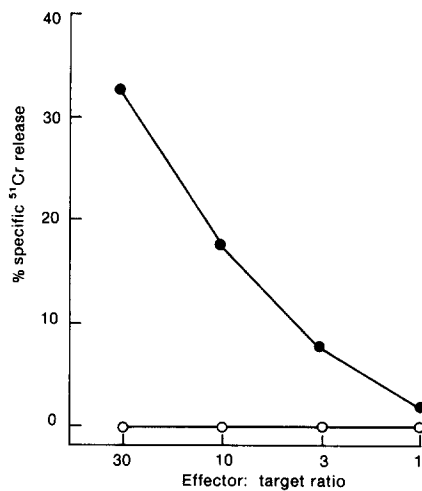


FIGURE 3. Alloantigen-specific killing effect of LL line. Killing effect of the LL line was tested on BALB/c (●) or C3H (○) Con A blasts.

specific for the Lyt-2 antigen was unable to affect the proliferative response of the LL line to BALB/c stimulators over a wide dose range (data not shown). These results suggest that LL cells that lack both Lyt-2 and L3T4 molecules on their cell surface are specific for class I molecules and not class II structures.

CTL Activity of the LL Line. We next analyzed the CTL activity of the LL line using C3H and BALB/c Con A blasts as targets (Fig. 3). The LL line developed a dose-dependent specific lytic activity against BALB/c but not C3H Con A blasts. The surface phenotype of the LL line was confirmed by treating the LL line with anti-Lyt-2 mAb or anti-L3T4 mAb plus C. As expected, anti-Lyt-2 mAb or anti-L3T4 mAb and C had no effect on CTL activity of the LL line (data not shown).

The molecules involved in cytolytic activity by the bulk-cultured LL line and derived clones were examined. The lytic activity of the LL line was tested on Con A blasts derived from several mouse strains (Table III). The double-negative clone L3C7, as well as the original bulk line, lysed BALB/c (K^dD^d) and B10.D2

TABLE III
Specificity of killing by LL line and clones

Target cells	H-2					Percent specific killing by LL line and clones		
	K	A	E	S	D	LL line	L3C7	N1G8
C3H	k	k	k	k	k	-2.2	2.4	1.3
BALB/c	d	d	d	d	d	31.5	33.8	21.5
B10.BR	k	k	k	k	k	1.0	6.4	4.0
B10.D2	d	d	d	d	d	17.6	34.6	28.5
B10.A	k	k	k	d	d	-5.4	-1.5	26.0
B10	b	b	b	b	b	-2.1	0.7	4.0

CTL activity of the LL line, double-negative clone L3C7, and Lyt-2⁺ clone N1G8 was tested on Con A blasts from various strains. E/T ratio, 5:1 for LL line, 3:1 for L3C7 and N1G8 clones. Data expressed as percent specific ⁵¹Cr release.

TABLE IV
Effect of mAbs on CTL Activity of the LL Clone

Clone	E/T ratio	Killing	Percent inhibition by mAbs in culture					
			Lyt-2	L3T4	F23	K ^d D ^d	I-A ^d	I-E
		%						
L3C7	2	74.4	3	-4	100	97	9	12
N1G8	2.5	73.5	90	-6	1	7	-4	14
N1D5	17	43.4	100	16	73	-17	18	-4

The blocking effects of several mAbs on CTL activity of double-negative clone L3C7 and Lyt-2⁺ CTL clones N1G8 and N1D5 were examined. The BALB/c-derived B cell tumor A20 was used as a target. Antibodies were hybridoma culture supernatants enriched by ammonium sulfate precipitation. Antibody was added in culture at a final concentration of 3%.

(K^dD^d) Con A blasts but did not lyse C3H (K^kD^k), B10.BR (K^kD^k), B10.A (K^kD^d), or third-party B10 (K^bD^b) Con A blasts.

A blocking study on the lytic activity of the LL cells was undertaken (Table IV). The cytotoxic activity of the bulk LL line and clone L3C7 was inhibited by the addition of anti-H-2 K^dD^d mAb 34-1.2 but not by several anti-Ia antibodies. Normal C3H anti-BALB/c clones N1G8 (specific for H-2 L^d) and N1D5, which recognize a histocompatibility structure that maps to the right of H-2D^d (Yui, K., unpublished observations), were not inhibited by any of these antibodies. These results, in combination with the study of CTL activity against a panel of targets, demonstrated that both the bulk LL line and clones were specific for class I molecules.

The blocking studies further demonstrated that the LL clone was completely inhibited by anti-TCR antibody F23. A normal T cell clone N1G8, which lacks the F23 determinant on its TCR, provided a suitable negative control. Finally, although anti-Lyt-2 mAbs inhibited normal Lyt-2⁺ clones N1G8 and N1D5, no significant effects on the activity of the LL cells were noted. We tested various concentrations of these mAbs and consistently obtained the same results (data not shown). Thus, it is unlikely that Lyt-2 or L3T4 molecules are even transiently expressed on the T cell surface and clearly play no role in the recognition functions of the LL cells studied.

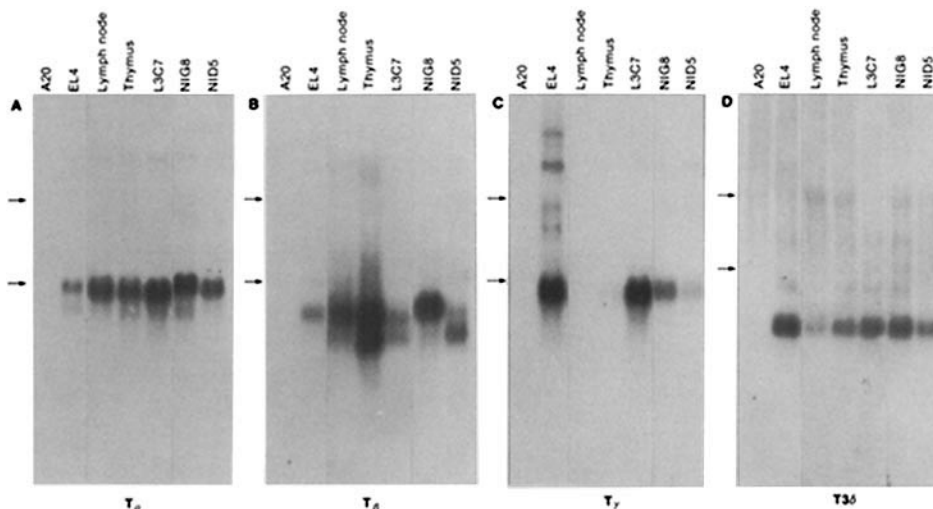


FIGURE 4. Northern blot analysis of T α , T β , T γ , and T3 δ RNA from a double-negative clone. 10 μ g of total RNA was extracted from mouse tissue and cell lines, separated on a 1.0% agarose gel, and blotted onto Genescreen plus. The same blotted filter was hybridized with ³²P-labeled T α (Nco I fragment of pHDS58), T β (Eco RI fragment of pUC25), or T γ (Pvu II fragment of pHDS205) cDNA probes. The T3 δ (pPGBC-9) cDNA probe was hybridized on a different blot. The positions of the 28S and 18S rRNA markers are indicated. A20, B cell lymphoma A20; EL4, thymoma EL4; lymph node, C3H-+/+ nylon-wool column-passed lymph node cells; thymus, C3H-+/+ thymocytes; L3C7, double-negative clone L3C7; N1G8, Lyt-2⁺ clone N1G8; N1D5, Lyt-2⁺ clone N1D5.

Expression of TCR RNA. We have previously shown that the bulk double-negative cell populations in lymph nodes of C3H-*gld/gld* mice express enhanced levels of T α , T β and T3 δ RNA. The level of γ gene transcript was comparable to that seen in normal peripheral T cells. In contrast to these observations, others have shown (43) that thymic double negative cells do not express α gene transcripts but do express β gene transcripts and enhanced levels of γ chain transcripts. In addition, it was of interest to determine whether functional double-negative T cells, which are barely detectable in normal mice and humans, express similar TCR RNAs when compared with normal Lyt-2⁺ or L3T4⁺ T lymphocytes.

Total RNA was purified from the B cell tumor A20, thymoma EL4, C3H/HeJ peripheral T cells, C3H/HeJ thymocytes, double-negative clone L3C7, and normal Lyt-2⁺ clones N1G8 and N1D5. Northern blots were hybridized with ³²P-labeled T α , T β , T γ , and T3 δ cDNA probes (Fig. 4).

Transcripts of the α and β chain of the TCR genes are of two types, the larger transcripts (1.6 kb for α and 1.3 kb for β) represent full-length transcripts of the V-(D)-J-C elements, whereas the smaller messages (1.3 kb for α and 1.0 kb for β) represent transcripts of (D)-J-C elements (44, 45). The double-negative clone expressed similar transcripts of the α chain gene when compared with normal peripheral T cells or normal clones. Similar amounts of both 1.3- and 1.0-kb transcripts of the β gene were expressed, but their level of expression was lower than normal peripheral T cells or normal clones. γ chain transcripts were also detected in the double-negative clone L3C7. The level of γ chain gene expression

in L3C7 was clearly higher than the normal clone N1G8 or N1D5, and comparable to that of the thymoma EL4. Finally, expression of T3 δ RNA was similar to that seen in normal CTL clones.

Discussion

In this study, we have successfully established cytolytic Thy-1⁺,Lyt-2⁻,L3T4⁻ T cell clones derived from the Lyt-2⁻,L3T4⁻ subset of lymph node cells from autoimmune C3H-*gld/gld* mice. These clones were IL-2 dependent in their growth and were shown to be specific for class I molecules. The inhibition of the responses of double-negative cell lines by anti-class I mAb was observed both on alloreactive proliferation and on specific killing of targets by the clones. These clones expressed and utilized α/β heterodimers of the TCR and thus are distinct from recently described (46–48) CD4⁻ and CD8⁻ cells that appear to express γ/δ heterodimers.

Lyt-2⁻ variants of originally Lyt-2⁺ CTL that maintain their CTL activity have previously been reported (49). However, these CTL lines were non-MHC specific and L3T4 expression was not studied. We did not add any reagents that induce mutation of cells nor did we use any selective pressure to maintain the double-negative phenotype. In addition, the double-negative phenotype was observed consistently from the initiation of the cultures. Therefore, we believe that the lack of Lyt-2 molecules on the clones is an intrinsic feature and, to our knowledge, this is the first demonstration of Lyt-2⁻,L3T4⁻ class I-specific CTL. The results obtained here clearly demonstrate further that Lyt-2 or L3T4 molecules are not obligatory for the induction, recognition, and killing activity of class I-specific CTL.

Budd and colleagues (50) reported that Lyt-2⁻,L3T4⁻ T cells from *lpr/lpr* lymph nodes or thymus cultured in the presence of phorbol ester and IL-2 displayed a broad cytotoxic range similar to NK or lymphokine-activated killer (LAK) cells. This type of cell may also be induced from *gld/gld* lymph node cells under certain culture conditions, since lymph node cells of the *lpr* and *gld* strains share many similar properties (51). It is not clear whether these two distinct Lyt-2⁻,L3T4⁻ populations, one specific for polymorphic determinants on MHC molecules and bearing α/β TCRs and another subset whose cytolytic activity is MHC nonspecific, represent the population at different stages of differentiation or represent cells of distinct lineages. In either case, the detection of these cell types suggests that Lyt-2⁻,L3T4⁻ cells in lymph nodes of *gld* mice are not homogeneous. This heterogeneity is also apparent in the adult Lyt-2⁻,L3T4⁻ thymocyte population that includes α/β TCR⁺ cells in addition to T3⁻ cells and γ/δ TCR⁺ cells (52, 53). Taken together, these results support the notion that the accumulating T cells in the peripheral lymphoid organs of mice with lymphoproliferative diseases may represent those that have escaped normal thymic differentiation processes (24).

Previous attempts to generate CTL from either *lpr* or *gld* mice have been unsuccessful (51, 54). In contrast, we were able to establish the existence of alloreactive CTL precursors (CTLp)¹ in the Lyt-2⁻,L3T4⁻ subset of *gld* mice. However, we observed that the frequency of alloreactive CTLp in the Lyt-2⁻,L3T4⁻ population is low when compared with C3H-+/+ T cells or unsepa-

rated C3H-*gld/gld* lymph node cells (data not shown). The explanation for the low frequency of CTLp in the Lyt-2⁻,L3T4⁻ subset is not obvious. One consideration is that there are several functionally distinct populations of double-negative cells and that the absolute number of CTLp in these populations is limited. In addition, the majority of double-negative cells in both *lpr* and *gld* lymph nodes appear to have abnormally phosphorylated TCR complex proteins (55). The relevance of abnormal phosphorylation patterns to functional activities or the lymphoproliferative defect of *gld* is, however, unclear. The second possibility to explain the low frequency is that although double-negative cells are fundamentally homogeneous, only a very limited number of the cells, whose TCRs by themselves have high affinity for allo-MHC, are generated. This idea is supported by the hypothesis that Lyt-2 or L3T4 molecules contribute to the affinity of the TCR for MHC (15, 16). Both of these mechanisms may contribute to the observed low frequency of MHC-specific T cells in double-negative populations.

Double-negative clones of the *gld* strain expressed both mature and truncated types of TCR RNAs. Transcripts of the α gene were comparable to those found in normal peripheral T cells, thymocytes, or T cell clones. Expression of the β chain gene was low when compared with peripheral T cells or when Lyt-2⁺ T cell clones were studied. However it is unclear whether these RNA patterns accurately reflect the population of functional double-negative cells in lymph nodes of the *gld* strain, because the number of clones analyzed was limited and also because cells cultured in vitro may show different patterns of gene expression from their counterparts in vivo. In contrast to the lower level of β gene expression, the γ gene transcript was abundant in double-negative clones. This high level of expression of the γ gene might correlate with the immature phenotype of these clones. The double-negative clone L3C7, however, does not express γ proteins on the cell surface (Yui, K., et al., unpublished observation). Therefore the γ transcript in this clone may be nonproductive, as are most γ transcripts detected in normal peripheral CTL (56, 57).

The correlation of expression and function of TCRs and their accessory molecules in the development of T cells in the thymus remains to be clarified. One fundamental hypothesis that derives from these studies is that development of antigen specificity for class I structures may occur before Lyt-2 gene activation. If this is the case then T cell programs may be activated first by class I or class II antigenic encounters in the thymus, leading to sequential expression of the Lyt-2 and L3T4 genes. It follows that these molecules may subserve some programmed function associated with the biology of those cell types, be it in terms of adhesion to targets or some other cellular activity. This hypothesis is supported by recent studies (52, 53) that have demonstrated the presence of α/β heterodimer-bearing cells in adult Lyt-2⁻,L3T4⁻ thymocytes.

We believe that phenotypically immature peripheral T cells in *gld* mice result from the abnormally programmed development of precursor cells in the thymus or other organs. Although these cells represent cell types that are present in much less significant numbers in the normal state, their further analysis will contribute to the understanding of T cell development and differentiation in both normal and abnormal states.

Summary

We report the first demonstration of Thy-1⁺,Lyt-2⁻,L3T4⁻ MHC-specific CTL clones derived from the Lyt-2⁻,L3T4⁻ subset of lymph node cells of C3H-*gld/gld* mice. These clones express α/β heterodimeric TCRs on the cell surface and specifically recognize class I molecules on target cells. Lyt-2 and L3T4 molecules are therefore not essential for the induction, recognition, and killing of antigen-specific CTL.

In addition, these studies suggest that antigen specificity development for class I structures may occur before Lyt-2 gene activation in the differentiation of T cells.

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