Lyt-2-/Lyt-3- VARIANTS OF A CLONED CYTOLYTIC T CELL LINE LACK AN ANTIGEN RECEPTOR FUNCTIONAL IN CYTOLYSIS*

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Detailed analysis of cytolytic T lymphocytes (CTL) at the molecular level has been hampered by the cellular heterogeneity intrinsic to most model systems studied. The development of cloned lines (1, 2) of CTL facilitates both biochemical (3) and genetic (this report) analyses of function.

CTL have been characterized by a distinctive cell surface antigenic profile that includes Thy-1, Lyt-2, and Lyt-3 (4). Several recent studies using alloantisera indicate that antibodies reactive with either Lyt-2 or Lyt-3, or with the product of a close-linked locus, are able to block CTL activity in the absence of complement (5, 6). Monoclonal antibodies directed against either Lyt-2 (7, 8) or Lyt-3 (J. A. Ledbetter and L. A. Herzenberg, personal communication) also have been found to block CTL activity in the absence of complement. These data imply a role for Lyt-2 and Lyt-3 in cytosis, but they cannot formally exclude that a molecule required for cytolysis activity merely is located near Lyt-2 and Lyt-3 on the cell surface. Antibody to Thy-1 does not block CTL activity in the absence of complement (7-9).

To investigate the role of Lyt-2 and Thy-1 in cytosis from an independent approach, we have generated, and analyzed the phenotypes of, variants of an especially lytic cloned CTL line (designated L3) which specifically lack either Lyt-2 or Thy-1. An analysis of these variants indicates that neither Lyt-2 nor Lyt-3 is responsible for the lethal hit, but suggests that Lyt-2 and/or Lyt-3 are required for an antigen receptor functional in cytosis. The data also suggest that the expression of Lyt-3 on the cell surface is not independent of the expression of Lyt-2. Finally the data indicate that Thy-1 plays no role in cytosis.

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Abbreviations used in this paper: CML, cell-mediated lympholysis; Con A, concanavalin A; CTL, cytolytic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; EMS, ethyl methanesulfonate; EDTA, N,N',N'-ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; LCA SF, supernate from Con A-stimulated rat spleen cell cultures; 2-ME, 2-mercaptoethanol; MLC, mixed leukocyte cultures; MLC SF, supernatant from secondary mixed leukocyte cultures; Mls, M locus; MOPS, morpholinopropane-sulfonic acid; PHA-P, phytohemagglutinin-P; SF, supernatant fluids.
Materials and Methods

*Animals.* Adult female DBA/2 (H-2\(^d\)) mice were obtained either from The Jackson Laboratory (Bar Harbor, Maine) or Laboratory Supply Co. (Indianapolis, Ind.). Adult female C57BL/6 (H-2\(^b\)) mice were obtained from The Jackson Laboratory. Adult female Lewis rats were obtained from Microbiological Associates (Walkersville, Md.).

*Culture Medium.* Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% heat-inactivated fetal calf serum (FCS) (KC Biological, Inc., Lenexa, Kans.), 5 \(\times\) 10\(^{-5}\) M 2-mercaptoethanol (2-ME), 10 mM morpholinopropane-sulfonic acid (MOPS), penicillin and streptomycin, and additional amino acids was used (10).

*Cloned CTL Line L3.* L3 is a cloned cytolytic T cell line of C57BL/6 (H-2\(^b\)) origin having cytolytic activity specifically directed against H-2D\(^d\) (2). It has a normal karyotype and is Thy-1.2\(^+\), LYT-1.2\(^-\), LYT-2.2\(^+\), and LYT-3.2\(^+\) (2). Both supernate from secondary mixed leukocyte culture (MLC) (MLC SF) (or another lymphocyte growth factor) and irradiated spleen cells are required for proliferation (1). L3 has been maintained in culture for 2 yr with no discernible change in phenotype.

L3 is passaged by transfer of 2 \(\times\) 10\(^6\) cells (100 \(\mu\)l, 7–8 d after previous transfer) to 3-ml tissue culture wells (17-mm diameter; Linbro 24-well plate, 76-033-05; Linbro Chemical Co., Hamden, Conn.). Each well contained 6.5 \(\times\) 10\(^6\) irradiated (1,400 rad) (model 143 cesium irradiator; JLS Shepherd & Associates, Glendale, Calif.) DBA/2 spleen cells and 33% supernate from concanavalin A (Con A)-stimulated rat spleen cells, (LCA SF) in a final vol of 1.5 ml. Culture plates were then incubated in a 37°C 5% CO\(_2\) humidified incubator.

*Cloned Amplifier T Lymphocyte Line L2.* L2 is a noncytolytic cloned M locus (Mlsa)-reactive amplifier T cell line of C57BL/6 origin. It is Thy-1.2\(^+\), LYT-1.2\(^-\), LYT-2.2\(^-\), and LYT-3.2\(^-\). The properties of L2 have been described in detail elsewhere (1, 2, 11). The protocol for passage of L2 is identical to that for L3, except that MLC SF is used in place of LCA SF.

*Production of Supernatant Fluids (SF).* The production of LCA SF and MLC SF have been described (2), but is briefly given here. (a) LCA SF: The supernate from a Con A (2.5 \(\mu\)g/ml) (Pharmacia Fine Chemicals, Uppsala, Sweden) -stimulated culture of Lewis rat spleen cells was collected after 48 h. (b) MLC SF: Secondary MLC were prepared by restimulating cells from primary C57BL/6 anti-DBA/2 MLC with irradiated DBA/2 spleen cells after 48 h of culture. After 48 h of culture, the supernate was collected.

*Antibodies.* The hybridoma antibodies AT83A (anti-Thy-1.2) (12), 2.43 (anti-Lyt-2.2) (7), 3.200 (anti-Lyt-2) (7), and 3.155 (anti-Lyt-2) (7) have been described. The anti-Lyt-3.2 hybridoma antibody 53.5.1 was kindly provided by Dr. J. A. Ledbetter and Dr. L. A. Herzenberg (Stanford University, School of Medicine, Palo Alto, Calif.) (13).

*Cell Variant Nomenclature.* The parental line is designated L3. The variants of L3 discussed here are designated L3:AT83A/1, L3:V4, L3:243/1, and L3:243/2; for convenience, the prefix "L3" will be dropped in subsequent references to the variants.

*Selection With Antibody and Complement.* Cells were incubated in the specified dilution of the antibody at room temperature for 30 min. After centrifugation, they were resuspended in the specified dilution of rabbit complement and incubated for 45 min at 37°C. The cells were then washed three times before being returned to culture.

*Treatment With Mutagen.* Each of the variants described here was derived from one of three independent cultures treated with the mutagen ethyl methanesulfonate (EMS) (Sigma Chemical Co., St. Louis, Mo.). The cultures were initiated with 2 \(\times\) 10\(^6\) L3/well. 5 d later, when the cell density had reached 6 \(\times\) 10\(^6\) L3/well. EMS was added to a final concentration of 150 \(\mu\)g/ml. 34 h later, cells were washed three times with DMEM and returned to culture as described under Results. The cloning efficiency of the EMS-treated cells relative to those cells which had not been exposed to EMS was 47%.

*Quantitative Immunofluorescence.* The quantitation of the cell surface staining was performed using a fluorescence-activated cell sorter (FACS III; B-D FACS Systems, Mountain View, Calif.) (14). Debris was removed from cell preparations before staining by Ficoll-Hypaque gradient centrifugation (15). For each histogram, 10\(^6\) viable cells as detected by forward-angle light scattering (1°–15° included angle) were analyzed (14). The fluorescence intensity units on the histograms are linear.
The procedure for indirect immunofluorescence staining of cells has been previously described (14). Hybridoma antibodies AT83A and 3.155 were coupled with biotin-succinimide (Biosearch, San Rafael, Ca.) using the technique described by Bayer and Wilchek (16). This permitted fluorescein-conjugated avidin (Vector Laboratories, Burlingame, Calif.) to be used as the second step in the indirect staining protocol. The second step for hybridoma antibody 53.5.1 was a fluorescein-conjugated rabbit anti-mouse Ig (17). For each stain, 10^6 cells were used and these were maintained at 0°C throughout the staining procedure.

**Assay for Cell-mediated Lympholysis (CML).** Cytolytic activity was measured as ^51^Cr release from labeled target cells. (a) To screen the microwells of a cloning plate (6.4-mm diameter; Costar 3596; Costar, Data Packaging, Cambridge, Mass.) for specific cytolytic activity, such as was done to select for V4, a microcytotoxicity assay using 500 target cells was employed (18). (b) The conventional cytotoxicity assay using 5,000 target cells is essentially as previously described (19), except that centrifugation for 60 sec at 200 g preceded the 3.5-h incubation at 37°C. This assay was used to compare the cytolytic activity of the variants with that of L3. When either Con A or phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, Mich.) was used in conjunction with this assay, the final lectin concentration was 10 μg/ml.

**Assay for Anti-Lyt-2.2 (2.43) Blocking of Cytolytic Activity in the Presence or Absence of Con A.** L3 cells were treated either with monoclonal antibody 2.43 (anti-Lyt-2.2) or with 20% FCS in DMEM for 30 min at 5°C. The cells were then assayed in a short-term ^51^Cr-release assay (7) for cytotoxicity against P-815 tumor target cells in the presence (10 μg/ml) or absence of Con A. EDTA (10 mM final concentration) was added after 30 min incubation with P-815 targets at 37°C. Amount of ^51^Cr released into the supernate was determined at 2 h (total) incubation.

**Targets.** P-815 is a tumor cell line of DBA/2 (H-2^d^) origin. EL4 is a tumor cell line of C57BL/6 (H-2^b^) origin. AKR-A is a tumor cell line of AKR (H-2^k^) origin. These lines were maintained in tissue culture.

**Results**

**Derivation of Variant V4.** After treatment with EMS for 34 h, trypan blue analysis indicated an average of 1.5 × 10^6 cells/well for two wells not exposed to EMS and an average of 8.5 × 10^6 cells/well for two wells exposed to EMS. Cells from these wells were cultured at limiting-dilution to determine the relative cloning efficiency of the EMS-treated cells. 10 d after initiating the culture, microwells were scored for proliferating cells and then tested for specific lysis of P-815 in a microcytotoxicity assay. V4 was selected as a well from the EMS-treated group that contained proliferating cells that had no demonstrable specific killing. For the particular dilution which yielded V4, 9 of 16 of the wells had proliferating cells.

**Derivation of Variants 243/1 and 243/2.** 32 wells that contained L3 cells were treated with EMS as described in Materials and Methods. 12 d after treatment, during which time the cultures were divided twice, the cells were harvested (12 × 10^6 total) and treated with 2.0 ml of undiluted hybridoma culture supernate 2.43 followed by 1.0 ml of a 1:5 dilution of rabbit complement. Trypan blue analysis indicated 97% killing. The surviving cells were placed in culture for eight days, after which this population (comprised of 1 × 10^6 cells) was again treated with 2.5 ml of undiluted hybridoma culture supernate 2.43 followed by 1.0 ml of a 1:5 dilution of rabbit complement. Trypan blue analysis indicated 95% killing. The treated cells were then transferred to 72 wells of a cloning plate. Thirteen days later, 2 of 72 microwells were positive for growth. These two proliferating lines were designated 243/1 and 243/2. The estimated mutation frequency was 3 × 10^-7.

**Derivation of Variant AT83A/1.** Another 32 wells that contained L3 cells were treated with EMS as described in Materials and Methods. 12 d after treatment, during which time the cultures were divided twice, the cells were harvested (13 × 10^6
and treated with 2.0 ml of a 1:20 dilution of hybridoma culture supernate AT83A followed by 1.0 ml of a 1:5 dilution of rabbit complement. Trypan blue analysis indicated 95% killing. The surviving cells were placed in culture for 8 d, after which this population (comprised of 3 × 10⁶ cells) was again treated with 6.0 ml of a 1:20 dilution of hybridoma culture supernate AT83A followed by 2.5 ml of a 1:5 dilution of rabbit complement. Trypan blue analysis indicated 60% killing. AT83A/1 was established from an aliquot of the surviving cells.

The estimated mutation frequency was 1 × 10⁻⁶.

FACS analysis (Fig. 1) and subsequent antibody-and-complement-mediated ⁵¹Cr-release assays, using monoclonal antibody AT83A, over a period of 2 months (data not shown) suggest that AT83A/1 represents a uniformly Thy-1⁻ population of cells. AT83A/1 is currently being cloned.

Surface Phenotype of the Variants. The surface phenotype of the variants was initially probed using an antibody-and-complement-mediated ⁵¹Cr-release assay (data not shown). Monoclonal antibodies AT83A, 2.43, and 3.200 were used. The parental line L3 was lysed by each of these antibodies. On the other hand, AT83A/1 was found to be specifically insensitive to AT83A. V4, 243/1, and 243/2 were found to be specifically insensitive to 2.43 and 3.200. At the level of this assay, therefore, it appeared that lines had been derived which had lost specifically either Thy-1 or Lyt-2.

A more rigorous analysis of the surface phenotype was provided by quantitative fluorescence using the FACS. Such an analysis is shown in Figs. 1, 2, and 3. The cells were stained with hybridoma antibodies recognizing either Thy-1.2, Lyt-2, or Lyt-3.2.

From Fig. 1, it is apparent that anti-Thy-1.2 stained L3, but did not stain AT83A/1. The patterns of reactivity for V4, 243/1, and 243/2 with anti-Thy-1.2 were comparable to that for L3 (data not shown).

The quantitation of the cell surface expression of Lyt-2 by the parental line L3 and the variant lines derived from it is shown in Fig. 2. It is apparent that anti-Lyt-2 stained L3, but did not react with the amplifier line L2 which had previously been

![Fig. 1. Expression of cell surface Thy-1.2. Variant AT83A/1 was derived from L3 as described in Results. Incubation of cells with biotin-succinimide-coupled monoclonal antibody AT83A (anti-Thy-1.2) was followed by staining with fluorescein-conjugated avidin. The FACS III was used to quantify the immunofluorescent staining.](image-url)
Fig. 2. Expression of cell surface Lyt-2. Variants AT83A/1, V4, 243/1, and 243/2 were derived from L3 as described in Results. L2 is a noncytolytic cloned amplifier T lymphocyte line. Incubation of cells with biotin-succinimide coupled monoclonal antibody 3.155 (anti-Lyt-2) was followed by staining with fluorescein-conjugated avidin. The FACS III was used to quantify the immunofluorescent staining.

Fig. 3. Expression of cell surface Lyt-3.2. Variants AT83A/1, V4, 243/1, and 243/2 were derived from L3 as described in Results. L2 is a noncytolytic cloned amplifier T lymphocyte line. Incubation of cells with monoclonal antibody 53.5.1 (anti-Lyt-3.2) was followed by staining with fluorescein-conjugated rabbit anti-mouse Ig. The FACS III was used to quantify the immunofluorescent staining.

shown to be Lyt-2− (2). The pattern of reactivity for AT83A/1 was comparable to that for L3. On the other hand, the staining profile for V4 was comparable to that for L2; that is, anti-Lyt-2 did not react with V4. 243/1 and 243/2 bound slightly more anti-Lyt-2 than did either L2 or V4. From the intensity of fluorescence, the amount of Lyt-2 on the surface of 243/1 and 243/2 was estimated to be <10% of that found on the surface of the parental line L3.

From Fig. 3, it is apparent that results obtained with anti-Lyt-3.2 mirrored those obtained with anti-Lyt-2 for all cell lines tested. The parental line L3 was positive, whereas L2 and V4 did not bind the anti-Lyt-3.2. Lines 243/1 and 243/2 showed fluorescence slightly above background, but exhibited <10% of the cell surface Lyt-3.2 expressed on L3.
Cytolytic Activity of the Variants. The parental line L3 specifically lyses targets that express H-2D\textsuperscript{d} alloantigen (2); however, it can be made to lyse irrelevant targets (those not expressing H-2D\textsuperscript{d}) by the appropriate concentration of either Con A or PHA-P. These lectins have been shown to allow the expression of cytolytic activity in the absence of a specific receptor-antigen interaction (20).

The cytolytic activity of AT83A/1 is similar to that of the parental line L3 (data not shown). It specifically lyses P-815 (H-2\textsuperscript{d}) target cells as efficiently as does L3. The irrelevant targets EL4 (H-2\textsuperscript{b}) and AKR-A (H-2\textsuperscript{k}) are lysed only in the presence of Con A or PHA-P.

The behavior of variant V4 is very different from that of L3 in that it appears to have lost the capacity to recognize the appropriate antigen. Specifically, in the absence of Con A, V4 lysed the relevant target P-815 at least 100 times less efficiently than does the parental line L3 (Fig. 4). However, in the presence of Con A, the lytic activity against P-815 is comparable to that of L3. Apparently the intrinsic cytolytic activity of V4 is comparable to that of L3, but V4 is defective in directing this cytolytic activity to the appropriate target. V4 lysed the irrelevant targets EL4 and AKR-A only in the presence of Con A or PHA-P (data not shown).

The behavior of variant 243/1 is similar in general to that of V4 in that it too is defective in lysing the relevant target P-815. However, in the presence of Con A, the lytic activity of 243/1 against P-815 is much less than that of L3 and V4 (Fig. 5). The behavior of 243/2 is essentially identical to that of 243/1. The basis for this difference between variant V4 and variants 243/1 and 243/2 has not yet been determined, but it indicates that line V4 is not identical to the lines 243/1 and 243/2.

Blocking of L3 Cytolytic Activity by Monoclonal Antibody 2.43 (Anti-Lyt-2.2) Can be Circumvented by Inclusion of Con A in the Assay. Monoclonal antibody 2.43 blocks the cytolytic activity of L3 against the relevant target P-815 in the absence of complement (7). It was of interest to examine whether this blocking is observed when Con A is included in the assay. The results given in Fig. 6 indicate that it is not. Con A-mediated lysis of the irrelevant target EL4 by L3 also is not blocked by antibody 2.43 (data not shown). Antibody 2.43 therefore appears to block receptor-mediated, but not lectin- (Con A) mediated, cytolysis by L3.

![Fig. 4. Cytolytic activity of variant V4 (○) compared with that of parent L3 (●). Cytolysis was measured in a 3.5-h \textsuperscript{51}Cr-release assay with P-815 tumor target cells, with (---) (10 \textmu g/ml) or without (−−−) Con A.](image-url)
Fig. 5. Cytolytic activity of variant 243/1 (△) compared with that of parent L3 (○). Cytolysis was measured in a 3.5-h 51Cr-release assay with P-815 tumor target cells, with (···) (10 μg/ml) or without (—) Con A.

Fig. 6. Blocking of L3 cytolytic activity by monoclonal antibody 2.43 (anti-Lyt-2.2) can be circumvented by inclusion of Con A in the assay. L3 cells were treated either with antibody 2.43 (○) or with medium alone (■) and then assayed in a short-term 51Cr-release assay for cytotoxicity against P-815 tumor targets in the presence (···) (10 μg/ml) or absence (—) of Con A.

Discussion

To investigate the role of Lyt-2 and Thy-1 in cytolysis, we have generated, and characterized the cytolytic activity of, variants of the cloned CTL parental line L3 that specifically lack either Lyt-2 or Thy-1. Collectively, the data indicate that neither Lyt-2 nor Lyt-3 is required for the lethal hit, but suggest that Lyt-2 and/or Lyt-3 are required to direct this lethal hit to the appropriate target. The data also suggest that the expression of Lyt-3 on the cell surface is not independent of the expression of Lyt-2. The results also indicate that Thy-1 is not required for cytolysis. Finally, the data presented here demonstrate the feasibility of analyzing CTL function by genetic manipulation of cell lines derived by the procedure used for L3. These points will be expanded upon below.

Variant AT83A/1 has no detectable Thy-1 on its surface. At this point, it is unclear which of the complementation classes of Thy-1− mutants is/are represented by AT83A/1 (21). In any case, these cells lyse P-815 specifically as well as does the parental line L3. This indicates that Thy-1 is not required for cytolysis.

The precise nature of the lesions leading to the Thy-1− and Lyt-2−/Lyt-3− pheno-
types is unclear, although biochemical (3) and genetic analyses should be informative. Specifically, it is unclear how pleiotropic the mutations are. However, it is important to note that the Thy-1- line expresses Lyt-2 and Lyt-3, and the Lyt-2- /Lyt-3- lines express Thy-1. Thus, expression of one set of cell surface antigens characteristic of subpopulations of T lymphocytes is not linked to expression of another cell surface antigen characteristic of all T lymphocytes. Also, FACS analysis using monoclonal antibodies indicates that V4 and L3 express to a comparable extent both T200 and another high-molecular-weight antigen which is present on activated T lymphocytes (M. Sarmiento, manuscript in preparation). Finally it is important to note that the retention of specific killing activity by the Thy-1- line indicates that the loss of specific cytolytic activity by the Lyt-2- /Lyt-3- lines is not a result of a nonspecific effect of EMS mutagenesis.

Previous observations that anti-Lyt-2 and anti-Lyt-3 antibodies block CTL activity in the absence of complement did not resolve whether the effect of antibody was on the recognition or the lethal hit stage of cell-mediated cytolysis. The variant cells provide discriminating evidence. Variant V4 demonstrates that the cytolytic deficiency of a Lyt-2- /Lyt-3- CTL variant can be entirely circumvented through employment of conditions (lectins) that do not require a specific receptor-antigen interaction. This places any role of Lyt-2/Lyt-3 in cytolysis before the lethal hit, in the recognition stage (22). The concomitant loss of specific killing and the Lyt-2- /Lyt-3- molecule(s) by at least two independent variant lines, selected for on the basis of different criteria, strengthens this interpretation. So does the observation that monoclonal anti-Lyt-2.2 antibody in the absence of complement blocks receptor-mediated, but not Con A-mediated, cytolysis by L3. Taken together, these data argue strongly that Lyt-2 and/or Lyt-3 are required for an antigen receptor functional in cytolysis. Supporting this interpretation are preliminary investigations of conjugate formation by the method of Glasebrook (23), which suggest that whereas L3 and AT83A/1 form conjugates with P-815 with comparable efficiency, V4 does not form conjugates (D. P. Dialynas, unpublished observations). The less efficient lectin-mediated cytolysis by 243/1 and 243/2 relative to that by V4 may be a result of the lesion(s) of the former extending either to a deficiency of the appropriate carbohydrate on the cell surface or to a functional loss of unidentified other component(s) required for cytolysis.

The results given here also indicate that at least two independent variant lines concomitantly lost both Lyt-2 and Lyt-3. In the case of 243/1 and 243/2, selection with anti-Lyt-2 monoclonal antibody and complement resulted in the loss of Lyt-3 but not of the other unselected marker Thy-1. Recent FACS analyses using monoclonal antibodies indicate that the parental line L3 expresses the same number of Lyt-2 and Lyt-3 determinants on the cell surface (J. A. Ledbetter and L. A. Herzenberg, personal communication). Collectively these data argue forcefully for a model in which the expression of Lyt-3 on the cell surface is in some way tightly coupled to the expression of Lyt-2 on the cell surface. This is compatible with recent speculation, arising out of an analysis of the structures recognized by anti-Lyt-2 and anti-Lyt-3 antibodies, that either the Lyt-2 and Lyt-3 determinants exist on the same molecule or the Lyt-2 and Lyt-3 molecules are associated as a dimeric complex (24, 25).

The results presented here suggest a model (5, 8) in which Lyt-2/Lyt-3 corresponds to a constant domain of the antigen receptor on the parental line L3. However, the
data cannot discriminate between a model in which the receptor specificity information is associated with the Lyt-2/Lyt-3 molecule(s) or one in which it is associated with another, independent molecule on the cell surface (in which case Lyt-2/Lyt-3 might serve to efficiently couple antigen recognition with cytolysis). The Lyt-2\(^{-}\)/Lyt-3\(^{-}\) variant lines described here, particularly V4, should facilitate experiments that address this question.

Summary

To investigate the role of Lyt-2 and Thy-1 in cytolysis, we have generated, by ethyl methanesulfonate mutagenesis and selection, variants of the cloned cytolytic T lymphocyte line L3 that specifically lack either Lyt-2 or Thy-1. An analysis of these variants indicates that neither Lyt-2 nor Lyt-3 is responsible for the lethal hit, but suggests that Lyt-2 and/or Lyt-3 are required for an antigen receptor functional in cytolysis. The data also suggest that the expression of Lyt-3 on the cell surface is not independent of the expression of Lyt-2. Finally the data indicate that Thy-1 plays no role in cytolysis.

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