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Research Article

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Effector Mechanisms in Organ-specific Autoimmunity

I. Characterization of a CD8⁺ T Cell Line That Mediates Murine Interstitial Nephritis

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Abstract

To further investigate mechanisms of cell-mediated tissue destruction in an organ-specific autoimmune disease, we have established and characterized a nephritogenic CD8⁺ T cell line. This target antigen-specific effector T cell line, M52, was derived from bulk populations of CD8⁺ T cells isolated from susceptible animals immunized to produce anti-tubular basement membrane (α TBM) disease. Our studies show that M52 retains the phenotypic and functional characteristics of nephritogenic T cells induced in vivo. M52 mediates antigen-specific delayed-type hypersensitivity (DTH) responses to the target antigen 3M-1, it is cytotoxic to 3M-1-expressing renal tubular epithelial cells in vitro, and it adoptively transfers interstitial nephritis to naive syngeneic recipients. Clonal analysis of these nephritogenic CD8⁺ T cells reveals distinct functional phenotypes within the M52 cell line. We have isolated a cytotoxic CD8⁺ clone, M52.26, which is not DTH-reactive to 3M-1, and multiple DTH-reactive clones which mediate less efficient cytotoxicity to 3M-1-expressing target cells. Cytofluorographic analysis of four randomly selected clones reveals $\alpha\beta$ T cell receptor expression. Further characterization of these functionally distinct CD8⁺ T cell clones will help to define their respective roles in mediating tubular epithelial cell injury and the inflammatory lesion of autoimmune interstitial nephritis. (*J. Clin. Invest.* 1991; 88:408–416.) **Key words:** autoimmunity • cell-mediated immunity • anti-tubular basement membrane disease • cytotoxicity • nephritogenic T cells

Introduction

Major advances in cellular and molecular immunology over the past decade have resulted in increasingly sophisticated analyses of the T cell repertoire in experimental autoimmune diseases (1–3). We have been interested in using models of autoimmune interstitial nephritis to understand immune system recognition of native renal antigens and the mechanisms by which activated lymphocytes elicit renal injury. One model studied extensively is anti-tubular basement membrane

(α TBM) disease in inbred mice (4–7). This form of progressive renal injury is produced by immunizing susceptible strains of mice (H-2K^d) with collagenase-solubilized renal tubular antigens (SRTA)¹ in CFA. By 2 wk after immunization, α TBM Abs are detectable in the kidney and after 6–8 wk mononuclear cells appear within the renal interstitium (4). The interstitial infiltrate becomes more intense with time and eventually results in tubular atrophy and interstitial fibrosis, with the functional consequence of progressive renal insufficiency.

The target antigen of both human and experimental α TBM disease has been purified (8, 9). This glycoprotein antigen, called 3M-1, displays conserved antigenicity across mammalian species. In the kidney 3M-1 is synthesized by proximal tubular epithelial cells (8) and is largely secreted into the extracellular matrix in close proximity to the tubular basement membrane. 3M-1 is also expressed on the proximal tubular cell membrane, albeit in lesser amounts than present in the extracellular matrix (10). As the cDNA sequencing of this glycoprotein has revealed five distinct sequences at the 5' end, the membrane-associated and matrix proteins may well be separate 5' isoforms of the 3M-1 antigen (11).

Previous studies have demonstrated that there are two distinct 3M-1 reactive T cell subsets found within the interstitial infiltrate of α TBM disease (12). Both subsets elicit an inflammatory interstitial lesion following adoptive transfer, but under quite different conditions (6, 13). A CD4⁺, class II-restricted (I-A^b) T cell subset adoptively transfers interstitial nephritis 6–10 wk after intravenous injection into naive syngeneic hosts (13). The CD8⁺ class I-restricted (H-2K^d) subset from immune mice acutely induces disease within 3–5 d, if transferred directly under the renal capsule (6). Subcapsular transfer of the CD4⁺ immune T cells does not induce interstitial disease (6). Previous studies have also demonstrated that the delayed-type hypersensitivity (DTH) response to 3M-1 is a valid assay of CD8⁺ effector T cell function, and is not mediated by CD4⁺ T cells in susceptible mice (6). Tubular antigen-specific DTH responses are demonstrable after antigen challenge following either subcutaneous immunization with renal tubular antigen (RTA) in CFA or intravenous transfer of immune T cells (6). In addition, CD8⁺ T cells that mediate DTH can be isolated from the interstitium of diseased animals. From this, and other studies (13–15), we have concluded that the CD8⁺ T cells are the ultimate effector T cells of this disease.

This work was presented in part at the annual meeting of the American Association of Immunologists, New Orleans, LA, June 1990, and at the American Society of Nephrology, Washington, DC, December 1990.

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1. *Abbreviations used in this paper:* APC, antigen-presenting cells; C β , constant region of the TcR β chain; DTH, delayed-type hypersensitivity; E/T, effector/target; MCT, murine proximal tubular epithelial cell line; MTT, 3(4,5-dimethylthiazol-2-yl)2,5 diphenyltetrazolium bromide; PPD, purified protein derivative; P1, P2, synthetic peptide fragments of 3M-1; RTA, renal tubular antigen; SRTA, soluble RTA; TcR, T cell receptor; TFB, murine interstitial fibroblast cell line; 3M-1, the nephritogenic moiety in SRTA; V, variable.

To evaluate more precisely the mechanisms of interstitial damage by CD8⁺ effector cells, we have developed a number of immunologic reagents to facilitate our analysis of T cell–epithelial cell interactions. MCT is a murine proximal tubular epithelial cell line established from microdissected tubules of naive SJL mice (10). This cell line expresses abundant cell-surface class I MHC as well as small amounts of class II, both of which can be augmented with IFN- γ (16). MCT cells secrete an extracellular matrix consisting primarily of laminin, types IV and V collagen, with lesser amounts of types I and III collagen. MCT cells also synthesize and secrete the target 3M-1 antigen of α TBM disease (10). Since MCT cells can synthesize both 3M-1 and class I MHC antigens, we anticipated that they may coexpress 3M-1 peptide fragments with class I MHC determinants as a complex which would be recognized by 3M-1–reactive CD8⁺ T cells. This report details our initial characterization of M52, a CD8⁺ effector T cell line which transfers interstitial nephritis, and its interaction with tubular epithelium.

Methods

Mice. SJL mice (H-2^s) were purchased from the Jackson Laboratory, Bar Harbor, ME.

Antigens. Rabbit renal tubular basement membranes were isolated by a differential sieving technique, sonicated, lyophilized, and stored at –20°C (4). Lyophilized RTA was emulsified in CFA at antigen concentration of 4 mg/ml for both RTA and purified protein derivative (PPD). SRTA was made from these lyophilized membranes by collagenase digestion (17). The 3M-1 glycoprotein is isolated from SRTA using immunoaffinity chromatography with a monoclonal α TBM-Ab (8). The resultant filtrate, SRTA depleted of 3M-1 was also used to assess antigen specificity in certain experiments. P1 and P2, two synthetic peptides derived from the cDNA sequences of 3M-1, were manually synthesized by the simultaneous multiple peptide method of Houghten (18). Amino acid sequences of these peptides are as follows: P1, LLRRRHGDRRSTMSAEVP; P2, ASAEQKEMEDKVTSPKAEAA (11). PPD was obtained from Connaught Laboratories, Ltd., Willowdale, Ontario, Canada.

Isolation of T cell lines (M52 and M61). Draining lymph nodes were harvested from SJL mice 14 d after immunization with either 2 mg of RTA/CFA or CFA alone. Lymph nodes were finely minced into small pieces, and gently pressed through a mesh sieving screen. Cells were washed with PBS and then incubated with monoclonal α B cell Ab (19), monoclonal α CD4 Ab (from GK 1.5 hybridoma [20, 21]), and a mixture of rabbit and guinea pig C (4). Remaining viable CD8⁺ cells were plated at a density of 0.5×10^6 /well of a 24-well tissue culture plate. The cell line and clones were propagated by weekly passage with 20 μ g/ml antigen (SRTA, P1, or PPD), 20% MLA-144 supernatants as a source of IL-2 and other growth factors (22), and 5×10^6 irradiated (2,500 rads) syngeneic spleen cells. The M52 cell line was cloned by limiting dilution. T cells were plated at a density of 0.3 cells/well in a 96-well microtiter plate with irradiated syngeneic splenocytes, IL-2, and antigen (SRTA or P1). Wells with evidence of T cell growth were then transferred to 24-well plates and further expanded by weekly stimulation. The surface phenotype of the clones was verified by antibody-mediated cytotoxicity using either GK 1.5 or α CD8 (from hybridoma 3.168.8 [23]) with C. T cell culture medium consisted of RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, MD) supplemented with glutamine, antibiotics (penicillin, streptomycin, gentamicin), 10% de-complemented FCS, 5% NCTC-109 (Whittaker Bioproducts, Inc.), and 2×10^{-5} M 2-mercaptoethanol. Typically, wells became confluent at 4–5 d and were carried every 7 d. All T cells were cultured at 37°C in a 5% CO₂ incubator.

Murine kidney cell lines. MCT (SJL proximal tubular epithelial) cells were grown at 37°C and 5% CO₂ in DMEM (JRH Biosciences, Lenexa, KS) with 10% FCS (14). They were used for cytotoxicity assays

at confluence. The renal fibroblasts (TFB), also harvested from SJL mice, were grown under the same culture conditions as the MCT cells and also used at confluence.

Local adoptive transfer of DTH. The adoptive transfer of DTH has been described in detail (6). In brief, cultured T cells were washed and resuspended at 2.0×10^7 cells/ml in SRTA (1 mg/ml), PPD (1 mg/ml), 3M-1 (1 mg/ml), P1 (1 mg/ml), P2 (1 mg/ml), or PBS, and 25 μ l of each cell preparation was injected into the hind footpads of recipient mice (three to four mice per group). Footpad swelling as an index of DTH was measured 24 h later using a spring-loaded engineer's micrometer (model 7308; Mitutoyo/MTI Corp., Paramus, NJ). The magnitude of swelling was expressed as the mean increment between the footpad injected with cells in antigen and that injected with cells in PBS in inches $\times 10^{-3} \pm$ SEM. In some experiments, T cell subpopulations were selectively depleted by incubation with α CD8 or α CD4 MAb and a mixture of rabbit and guinea pig C before adoptive transfer. All footpad measurements were performed by an individual blinded to the experimental protocol.

Adoptive transfer of disease. These subcapsular transfers have been previously described (6). In brief, naive SJL mice were anesthetized, and 25×10^6 cultured lymphocytes in $\sim 75 \mu$ l of PBS were injected under the kidney capsule with a 30-gauge needle. This volume uniformly lifted the capsule off most of the parenchyma without bleeding. 7 d later the kidneys were harvested and longitudinally sectioned with preservation of the subcapsular cell layer. After fixation in 10% buffered formalin, the kidneys were paraffin embedded for staining with hematoxylin and eosin. The kidneys were then examined histologically for the presence of interstitial injury.

Assessment of renal disease. The stained kidney sections were coded for blind reading by two different grading scales. The severity was qualitatively assessed with a scale used in previous studies (6): 0 = no involvement from the subcapsular cell layer; 0.5 = trace pathologic changes of cellular involvement in a focal pattern in the outermost cortical tubular area; 1 = superficial, focal peritubular infiltration and tubular atrophy under the transferred cell layer; 2 = focal, deeply extending, heavy cellular infiltrates with peritubular damage and tubular atrophy. The sections were also graded by approximating the tubular layer cell depth of the most advanced infiltrating front of mononuclear cells. Each layer equalled a tubular diameter and was given one point. The data from both methods were expressed as a mean for each group \pm SEM.

Cytotoxicity assays. This method determines target cell viability (24, 25). Target cells, either MCT or TFB cells, were prepared from confluent cultures by light trypsinization (Sigma Chemical Co., St. Louis, MO) and washed once in appropriate serum-containing medium. Cells were then resuspended at 1.0×10^6 /ml in complete medium containing 0.5 μ g/ml mitomycin C (Sigma), vigorously vortexed, and 20- μ l aliquots were dispensed into 96-well flat-bottomed microtiter plates. 180 μ l of mitomycin C-containing medium (0.5 μ g/ml) was then added to each well. Target cells were allowed to attach by overnight incubation at 37°C, 5% CO₂. Effector T cells were harvested from day 5 cultures containing irradiated syngeneic feeders, IL-2, and antigen, and separated by Lympholyte (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada) extraction at room temperature. The interface cell layer containing viable T cells was washed with fresh medium and resuspended at 10×10^6 cells/ml. Appropriate aliquots were then added to the adherent target cells at effector/target (E/T) ratios of 5/1–100/1 in triplicate. Plates were incubated for 16–20 h at 37°C and 5% CO₂. Four wells containing target cells received no effector cells as a control for MCT cell viability and four wells containing only medium were used to control for nonspecific dye reduction. After effector cell incubation, T cells were washed from the microtiter wells with warmed (37°C) medium, and the wells were replated with 180 μ l of fresh medium. 20 μ l of 3(4,5-dimethylthiazol-2-yl)2,5 diphenyltetrazolium bromide (MTT) dye (Sigma) solution (5 mg/ml in PBS) was added to each well and the plate was incubated for 4 h at 37°C, 5% CO₂. After this incubation, medium and unreacted dye were aspirated from the wells and 180 μ l of 0.04 N HCl in isopropanol was added to solubilize the

formazan dye. In addition, 20 μ l of 3% SDS solution was added to each well to solubilize alcohol-induced protein precipitates. The OD at 570 nm of each well was then determined using a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The mean and standard deviation were determined for triplicate samples. The percent cytotoxicity was calculated by the following equation: % cytotoxicity = $(1 - \text{OD treated/OD control}) \times 100$.

Blocking antibody studies. These studies were conducted at an E/T ratio of 25/1. Cytotoxicity assay plates were set up as described above. Varying concentrations of monoclonal antibodies α CD8 (23), α CD4 (20, 21), α H-2K^a (26), α H-2K^b (27), or α Thy 1.2 (19) were added to either effector T cells or target cells (H-2K^a) before the experiment. After overnight incubation, plates were washed, and MTT was added to each well. The OD readings were conducted at 570 nm to determine cell viability. Results were then compared to control wells to which no antibody was added.

Southern hybridization studies. Liver and M52 clonal DNA were isolated with standard methods (28). Each sample, containing 10 μ g of genomic DNA, was digested to completion with a restriction enzyme (Hind III, Boehringer Mannheim Corp., Indianapolis, IN), separated on a 0.7% agarose gel, and transferred to a Zetabind[®] membrane (Cuno Laboratory Products, Meriden, CT). The blots were prehybridized and hybridized at 55°C in 0.5 M NaPO₄ buffer, 7% SDS, 1% BSA, 1 mM EDTA, 50 μ g/ml Poly A (Boehringer Mannheim), and 50 μ g/ml ssDNA. The blots were hybridized to a ³²P-labeled (random-primed) murine T cell receptor (TcR) C β probe (29) (sp act: 2.0×10^9 dpm/ μ g) for 16 h, and then washed three times for 30 min in 0.1 \times SSC, 0.1% SDS at 65°C. Autoradiograph exposures were obtained with intensifying screens at -70°C.

Cytofluorography. Cytofluorography was performed on M52 clones harvested from day 9 cultures. Cells were washed in PBS, and aliquots of 1×10^6 cells were resuspended in 50 μ l PBS with 0.1% BSA (staining buffer). FITC-conjugated antibodies, (α TcR [$\gamma\delta$] [30] and α TcR [$\alpha\beta$] [31]; PharMingen, San Diego, CA), were incubated with samples on ice for 20 min, and samples were washed three times with staining buffer. Stained cells were then fixed in 500 μ l of 4% paraformaldehyde in PBS. Fluorescence was recorded on a FACScan[®] cytofluorograph (Becton Dickinson & Co., Mountain View, CA). In each run, 10,000 live gated cells were analyzed.

Statistical analysis. Differences between experimental groups were determined by Student's *t* test, where appropriate. Cytotoxicity curves were evaluated using two-way analysis of variance (32).

Results

General characterization of the M52 cell line. Previous studies from our laboratory have demonstrated that adoptive transfer of bulk populations of CD8⁺, 3M-1-specific T cells results in a histologic lesion very similar to that seen after immunization with RTA in CFA (6, 14). The major histologic difference between disease caused by active immunization and adoptive transfer of immune cells is the absence, after adoptive transfer of CD8⁺ T cells, of α TBM-Abs. These CD8⁺ effector T cells also transfer DTH to the target antigen, a property that has facilitated their characterization (6). The M52 cell line was isolated from CD8⁺ T cells harvested from SJL mice immunized with RTA/CFA. Since long-term in vitro culture can alter T cell specificity and function, we performed a number of studies to assess whether cultured M52 cells retained the functional characteristics of immune CD8⁺ cells from immunized mice.

Table I displays DTH responses observed in syngeneic mice following local adoptive transfer of M52 cells. M52 cells transfer a DTH response to SRTA, but not to PPD, another antigen present in the immunizing preparation. This response was eliminated by pretreatment with α CD8 and C, but not with

Table I. CD8⁺ M52 Cells Transfer a DTH Response to SRTA

Donor cells*	Pretreatment [‡]	DTH response [§]	
		SRTA	PPD
M52	—	19.3 \pm 3.0	5.3 \pm 0.3
M52	C + α CD8	5.3 \pm 1.5	—
M52	C + α CD4	20.1 \pm 2.7	—
M52	C	20.7 \pm 2.4	—
M61	—	5.0 \pm 1.0	19.7 \pm 3.2
M61	C + α CD8	—	3.7 \pm 0.9
M61	C + α CD4	—	21.0 \pm 1.2
M61	C	—	18.3 \pm 0.9
—	—	3.7 \pm 0.9	—

* After 5 d in cultures containing irradiated syngeneic feeders, IL-2, and antigen, cells were harvested and injected into the footpads of recipient naive SJL mice. [‡] Before footpad injection, specific groups of harvested cells were pretreated with various monoclonal antibodies and C to define T cell subsets. [§] Values expressed are the mean of three mice in inches $\times 10^3 \pm$ SEM. These values represent the incremental difference in footpad swelling, measured 24 h after challenge, between the footpad that received 0.5×10^6 cells plus antigen (SRTA or PPD, 1 mg/ml) and that which received 0.5×10^6 cells and PBS.

^{||} *P* < 0.001 compared with no cells injected.

α CD4 and C, nor C alone. In addition, we generated another CD8⁺ cell line, M61, to have a control cell line for further investigations. This line was derived from a syngeneic animal immunized with CFA and, as shown in the fifth line of Table I, transfers a DTH response to PPD but not to SRTA. Selective depletion of M61 cells with MABs and C demonstrate that this DTH-reactive population is also CD8⁺ and CD4⁻.

Fine specificity of the M52 cell line. SRTA contains a mixture of 15–20 proteins by SDS-PAGE analysis (8). Although previous work has suggested that 3M-1 is the dominant immunogenic protein in this preparation, we confirmed the antigen specificity of M52 cells. Table II shows that M52 cells transfer a DTH response to SRTA or to the purified target antigen 3M-1, but not to SRTA depleted of 3M-1 by immunofluorescence chromatography (8). The cDNA encoding murine 3M-1 has been recently sequenced (11). Two nonoverlapping peptides derived from this sequence were also used as antigens in the local adoptive transfer of DTH with M52 cells. Other studies from our laboratory have demonstrated that P1, but not P2, is recognized by both a 3M-1-specific CD4⁺ T helper clone, as

Table II. M52 Cells Specifically Recognize 3M-1

Donor cells*	Injected antigen	DTH response [‡]
M52	SRTA	19.0 \pm 1.5
M52	3M-1	15.7 \pm 2.0
M52	SRTA - 3M-1	4.0 \pm 0.6 [§]
M52	P1	16.3 \pm 0.9
M52	P2	2.3 \pm 0.6 [§]
M52	PPD	5.3 \pm 0.3 [§]

* See * of Table I. [‡] See [§] of Table I. Values are the mean of three mice in inches $\times 10^3 \pm$ SEM. [§] *P* < 0.001 compared to M52 cells injected with SRTA.

well as a monoclonal α 3M-1 antibody (11). As seen in Table II, M52 cells recognize P1, but not P2. Although we have not ruled out the possibility that other regions of 3M-1 are immunogenic in SJL mice, the P1 fragment is emerging as an immunodominant epitope in this strain.

M52 cells elicit an inflammatory interstitial lesion after adoptive transfer. The M52 cell line was started from lymphocytes isolated from mice 2 wk after immunization, or before the onset of disease. We examined the *in vivo* function of the cell line by examining its ability to induce interstitial nephritis in naive recipients. In previous studies we have found that renal subcapsular transfer of immune T cells is a sensitive and reproducible means of determining the ability of discrete T cell subsets to elicit inflammation in the renal interstitium (6, 13, 14). To compare the nephritogenic potential of M52 cells with these previous studies, we used this same technique. 7 d after subcapsular injection, kidneys were harvested and evaluated by

light microscopy for both severity of injury and maximum depth of infiltrating cells (described in Methods). Panels *A* and *C* of Fig. 1 contrast the severe interstitial injury induced by the M52 line with the normal histology seen in Panels *B* and *D* after adoptive transfer of equal numbers of PPD-reactive M61 cells. Involved kidneys display focal mononuclear cell aggregates as well as mononuclear cells infiltrating "single file" between tubules. Other areas display marked tubular dilatation, without striking lymphocyte infiltration. The formal analysis of these groups is displayed in Table III. Interstitial lesions were not present in any recipients of M61 cells. In the cohort injected with C-treated M52 cells, one of the four kidneys revealed no evidence of disease, likely a result of a technical problem at time of injection. The other three kidneys in this group exhibited interstitial lesions similar in severity to the untreated M52 group. The nephritogenicity of M52 cells, like the DTH response, was also eliminated with α CD8 and C. In addition,

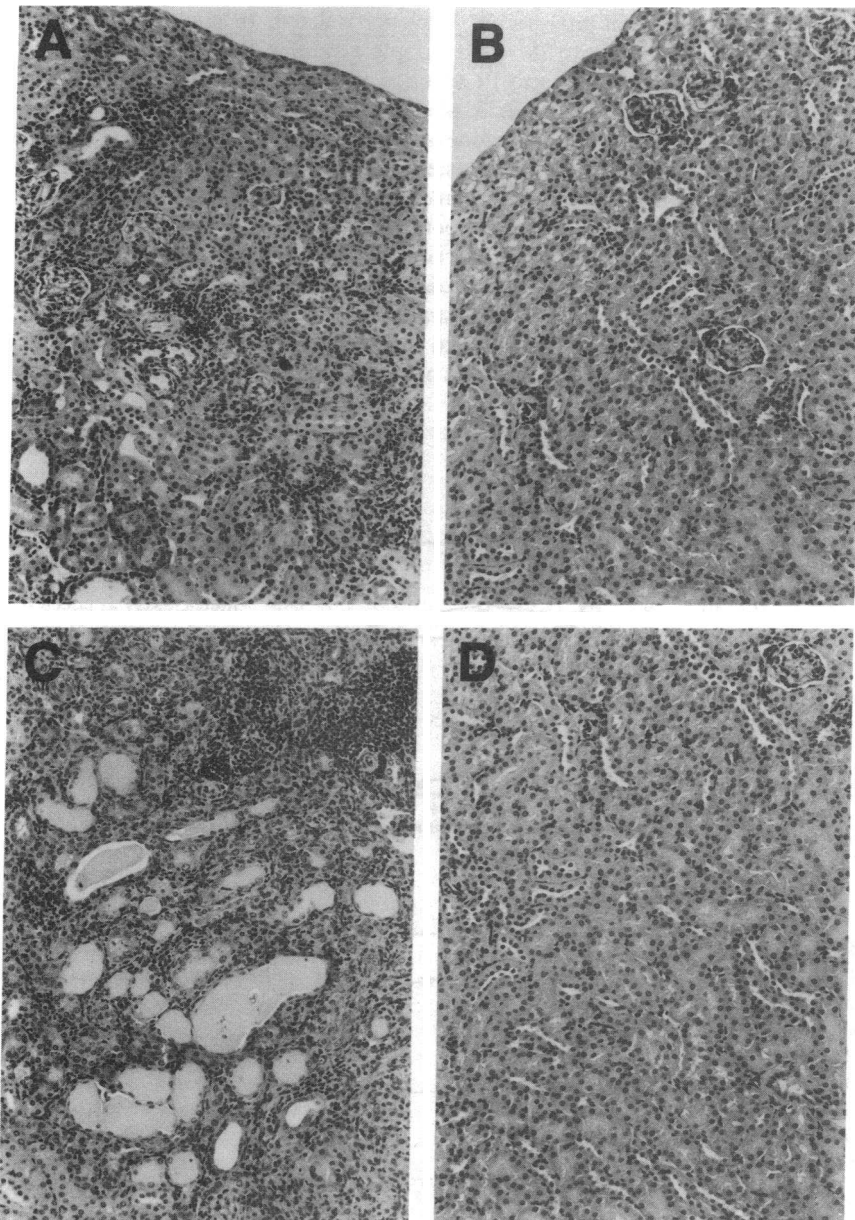


Figure 1. Subcapsular cell transfers. Cultured M52 cells (*A, C*) or control M61 cells (*B, D*) were injected under the kidney capsule of naive syngeneic mice. After 7 d the kidneys were harvested and sectioned for histologic grading. The interstitial lesion typically seen after transfer of M52 cells is seen in both *A* and *C*. Focal cortical areas of mononuclear cell infiltration are evident, as well as areas of tubular dilatation. Control M61 cells do not infiltrate the renal parenchyma, as shown in *B* and *D*. The tubules and interstitial architecture remain normal (*A-D*, $\times 200$).

Table III. M52 Cells Mediate Interstitial Nephritis After Subcapsular Transfer

Donor cells*	Pretreatment	Histology after subcapsular transfer [†]	
		Severity	Maximum depth
M52	—	1.8±0.3	17.0±3.9
M52	C	1.0±0.5	10.8±4.6
M52	C + α CD8	0.1±0.1	0.3±0.3
M61 [‡]	—	0	0

* Cultured cells were harvested from day 5 cultures containing irradiated syngeneic feeders, IL-2, and antigen. In some groups cells were pretreated with antibody and/or C before transfer. 25×10^6 cells in 0.075 ml of PBS were injected under the kidney capsule of naive SJL mice. ($n = 4$ for each group). [†] 7 d after transfer, the kidneys were harvested and sectioned for histologic grading as detailed in the Methods section. [‡] $n = 3$.

the morphologic characteristics and severity of M52-mediated interstitial lesions are indistinguishable from those seen after adoptive transfer of 3M-1-specific CD8⁺ cells taken directly from immunized animals (6, 13, 14). Despite long-term culture, M52 cells maintain their in vivo functional characteristics and specificity.

M52 cells are cytotoxic to syngeneic proximal tubular epithelial cells. CD8⁺ T cells are traditionally associated with cytotoxicity rather than DTH, a function typically mediated by CD4⁺ T cells. There are models other than α TBM disease, however, in which CD8⁺ T cells mediate adoptive transfer of DTH (33, 34). To further characterize the functional attributes of M52 cells we evaluated their cytotoxic potential against a renal proximal tubular epithelial cell line, MCT (10). Since MCT cells synthesize the 3M-1 glycoprotein, and express class I and small amounts of class II MHC glycoproteins on the cell surface, we reasoned they may be recognized by M52 cells. We employed a colorimetric assay designed for cytotoxic assays of adherent cells (as MCT is) (24, 25) to examine whether M52 was cytotoxic to MCT. As shown in Fig. 2, M52 cells display dose-dependent cytotoxicity toward MCT cells between E/T ratios of 5/1 and 75/1, above which 100% cytotoxicity is typically observed. A number of control studies were performed to examine the specificity of this interaction. CD8⁺ PPD-reactive M61 cells display minimal cytotoxicity to MCT, and M52 cells are not cytotoxic to cultured renal fibroblasts from SJL mice, which express class I MHC but not the target 3M-1 antigen (11,

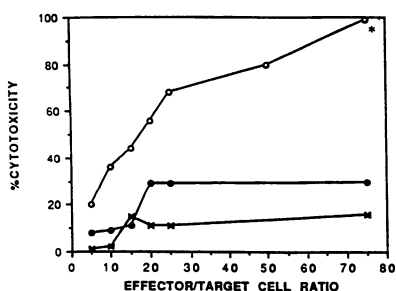


Figure 2. Quantitative assessment of M52-induced cytotoxicity. Adherent target cells (MCT or TFB) were incubated with effector cells (M52 or M61) for 16 h at 37°C and the percent cytotoxicity was determined from the amount of MTT formazan produced, as described in

Methods. M52/MCT (○), M52/TFB (×), and M61/MCT (●). Each data point represents the mean of triplicate samples. The asterisk denotes statistical significance of the curve ($P < 0.05$) from M52/TFB and M61/MCT based on the two-way analysis of variance.

35). These studies support the specificity of the M52-MCT interaction.

M52-mediated cytotoxicity is blocked by antibodies to CD8 or class I MHC. We examined whether cytolysis of MCT cells by M52 was a conventional cytotoxic T lymphocyte-target cell interaction in follow-up studies using blocking antibodies. These studies were carried out at an E/T ratio of 25/1, where a relatively small change in E/T ratio results in a significant change in cytotoxicity to MCT cells. As seen in Fig. 3, A and B, either α CD8 or α H-2K^s interfere with M52-mediated cytotoxicity, in a dose-dependent manner. α CD4, α H-2K^d, or α Thy 1.2, as a control antibody recognizing another cell-surface determinant on M52 cells, did not significantly interfere with M52-mediated cytotoxicity of MCT cells.

The M52 cell line is comprised of two functionally distinct CD8⁺ subsets. The functional studies described above raised the obvious question of whether DTH and cytotoxicity were mediated by distinct CD8⁺ clones or whether a single clone is capable of both functions. M52 was subcloned by limiting dilution (0.3 cells/well) and the resultant clones subjected to functional analysis. Of 17 clones isolated, 16 mediated DTH responses to 3M-1 after adoptive transfer. DTH responses of four such clones to P1 (peptide fragment of 3M-1) are shown in Table IV. M52.23, M52.28, and M52.34 are representative of the majority of clones analyzed thus far in that they adoptively transfer DTH responses to SRTA (data not shown) and also to P1. M52.26 is a functionally unique clone as it does not transfer a DTH response to P1, nor does it transfer DTH to intact antigen (data not shown). These four clones were also examined for cytotoxicity to MCT cells. As seen in Fig. 4, all four clones demonstrated cytotoxicity to MCT cells. These studies were repeated several times and consistently showed two distinct patterns of cytotoxicity. M52.26, the clone which

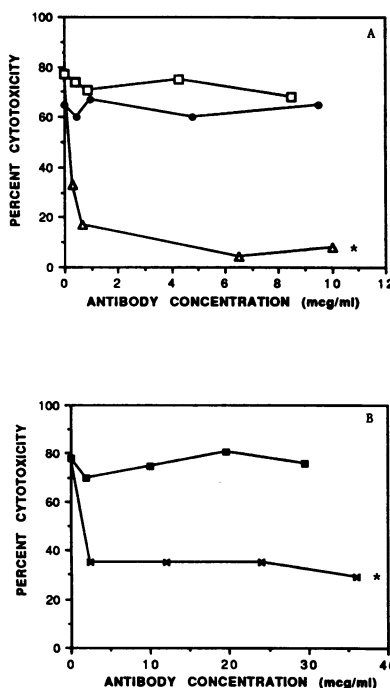


Figure 3. Blocking antibody studies of M52-induced cytotoxicity.

These cytotoxicity assays were performed with the MTT colorimetric assay at effector/target cell ratios of 25/1. (A) α CD8 (Δ), α CD4 (\square), or α Thy1 (\bullet) were incubated with effector M52 cells before their interaction with target MCT cells. (B) Anti-class I antibodies, either α H-2K^s (\times) or α H-2K^d (\blacksquare), were incubated with target MCT cells before the addition of effector M52 cells. Each data point represents the mean of triplicate samples. The asterisk denotes statistical significance of curves ($P < 0.05$) from control antibody curves (α CD4 or α H-2K^d) based on the two-way analysis of variance.

Table IV. Some M52 Clones Mediate DTH to Synthetic 3M-1 Peptides

Donor cells*	Injected antigen	DTH response [‡]
M52.23	P1	19.0±1.2 [§]
M52.26	P1	5.7±2.3
M52.28	P1	18.7±1.2 [§]
M52.34	P1	20.3±0.9 [§]
—	P1	4.3±0.3

* See * of Table I. [‡] See [§] of Table I. Values are the mean of three mice in inches × 10³±SEM. [§] P < 0.001 compared to no cells injected.

is not DTH-reactive, displayed more significant cytotoxicity at lower E/T ratios when compared to the three DTH-reactive clones ($P < 0.05$). M52.23, M52.28, and M52.34 all require higher E/T ratios than M52.26 to achieve comparable percent cytotoxicity, and even at E/T ratios of 50/1, kill only 40–50% of the MCT cells. The differences in DTH reactivity as well as the disparate cytotoxic effects of these clones suggest that there are functionally distinct clones within the M52 cell line.

M52 clonal TcR gene rearrangement. Given the common functional properties of the DTH-reactive clones we evaluated these cells for TcR- β gene rearrangement, to verify that they are distinct clones. The Southern blot analysis in Fig. 5 displays SJL liver and M52 clonal DNA, digested with Hind III and hybridized to a labeled C β probe. As seen in Fig. 5, Hind III digestion of germline DNA results in a 9.4-kb C β 1 fragment and a 3.0-kb C β 2 fragment. M52.23, M52.26, M52.28, and M52.34 display unique C β 1 fragments (8.3, 8.7, 9.0, and 12.6 kb, respectively) which differ from the germline configuration. Cell-surface expression of TcR on these clones was confirmed with cytofluorography. Fig. 6 demonstrates the expression of $\alpha\beta$ TcR, and absence of $\gamma\delta$ TcR, on these immunocompetent CD8⁺ M52 effector clones.

Discussion

The studies presented in this paper demonstrate that cultured M52 cells maintain the full spectrum of functions mediated by immune CD8⁺ T cells isolated from immunized mice. They induce antigen-specific cytotoxic and DTH responses, and are

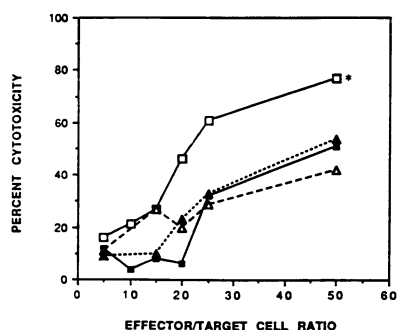


Figure 4. Cytotoxicity of M52 clones. Adherent target MCT cells were incubated with effector M52 clones for 16 h at 37°C and the percent cytotoxicity was determined as described in the Methods section. M52.23/MCT (—■—), M52.26/MCT (—□—), M52.28/MCT (---△---), M52.34/MCT (---▲---). Each data point represents the mean of triplicate samples. The asterisk denotes statistical significance of the curve ($P < 0.05$) from M52.23, M52.28, and M52.34 based on the two-way analysis of variance.

capable of infiltrating the kidney after adoptive transfer. M52 clones express cell surface $\alpha\beta$ TcR and segregate into two distinct groups by functional analyses. One subset is cytotoxic while the other is DTH-reactive and effects much less efficient cytotoxicity. We believe the pathophysiologic autoimmune T cell response resulting in α TBM disease comprises both functional subsets. Further studies will address this issue by delineating and comparing the types of interstitial injury induced by these functionally distinct clones.

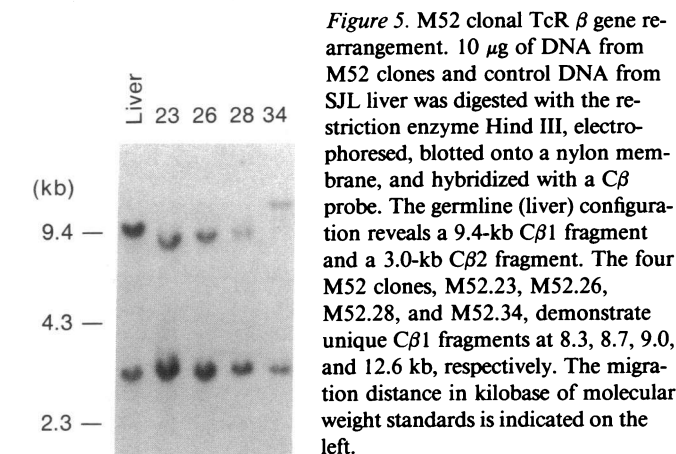


Figure 5. M52 clonal TcR β gene rearrangement. 10 μ g of DNA from M52 clones and control DNA from SJL liver was digested with the restriction enzyme Hind III, electrophoresed, blotted onto a nylon membrane, and hybridized with a C β probe. The germline (liver) configuration reveals a 9.4-kb C β 1 fragment and a 3.0-kb C β 2 fragment. The four M52 clones, M52.23, M52.26, M52.28, and M52.34, demonstrate unique C β 1 fragments at 8.3, 8.7, 9.0, and 12.6 kb, respectively. The migration distance in kilobase of molecular weight standards is indicated on the left.

These studies support our previous observations that class I-restricted CD8⁺ T cells are the ultimate effector cells in this model (6, 13). The ability of CD8⁺ T cells to recognize a renal epithelial cell that expresses the 3M-1 antigen is entirely consistent with a currently accepted model in which class I-restricted CD8⁺ T cells recognize antigen endogenously synthesized by a target cell (36). The ability of CD8⁺ T cells to mediate DTH responses to 3M-1 in local adoptive transfer assays does not fit this model, however, since presumably antigen-presenting cells (APC) in the footpad are processing and presenting exogenous 3M-1 antigen in association with class I MHC. Recent studies examining class I and II MHC-restricted pathways of ovalbu-

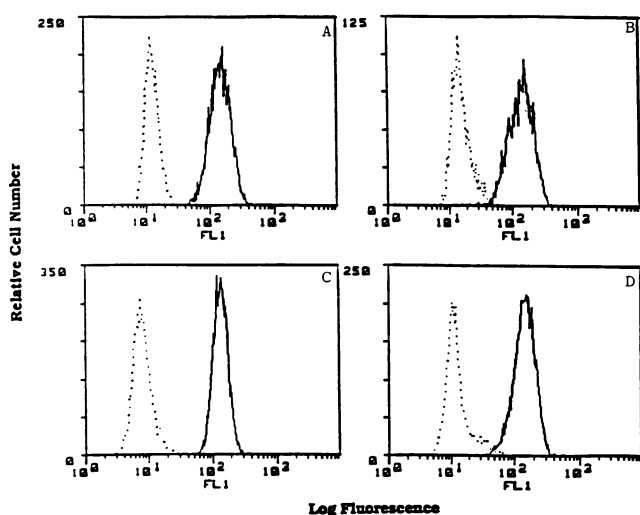


Figure 6. Cytofluorography of four M52 clones. Day 9 cultured M52 clones: (A) M52.23; (B) M52.26; (C) M52.28; (D) M52.34, were incubated with FITC-hamster anti-mouse TcR antibodies. $\alpha\beta$ TcR antibody (—), and $\gamma\delta$ TcR antibody (---).

min presentation, however, support the notion that exogenous antigens can be presented with class I MHC (37). CD4⁺ T cells, on the other hand, typically recognize exogenous antigen, processed by APC, in association with class II MHC (38). We have previously shown that 3M-1-specific DTH-reactive CD4⁺ T cells also expand after immunization to produce α TBM disease (12). Unlike the CD8⁺ effector cells, the CD4⁺ effector cells do not typically infiltrate the kidney to cause inflammatory interstitial nephritis, a functional deficit that can be largely attributed to low basal levels of class II MHC expression in kidney tubular epithelium. When class II MHC expression is increased with IFN- γ , CD4⁺ effector cells do infiltrate the kidney (16). In this setting of augmented renal class II expression, it is unknown whether epithelial cell 3M-1 *in vivo* is presented to CD4⁺ effector cells by tubular cells or other more traditional APC. Studies performed *in vitro*, however, suggest that tubular epithelial cells can present self-synthesized antigen to class II-restricted CD4⁺ T cell clones (35).

M52-mediated cytotoxicity of MCT cells can be inhibited by antibodies to CD8 or class I MHC, suggesting that the interaction of these autoimmune effector T cell clones with renal epithelium conforms to the conventional "rules" of cytotoxic T cell-target cell recognition (36). These findings should not be interpreted, of course, to mean that the TcR/CD8 interaction with a complex of class I MHC and target peptide on the MCT cell surface is the only important cell-cell interaction. In view of recent studies on lymphocyte-endothelial interactions, it is quite likely that the M52-MCT interaction is also strengthened by interactions between adhesion molecules and integrins (39, 40). It is also important to note that *in vitro* cytotoxicity studies largely overlook any role played by cytokines or antigen-specific immune reactants, which *in vivo* may serve to either augment or diminish the efficiency of the CD8⁺ effector cell-MCT interaction. IFN- γ and tumor necrosis factor- α , for example, have potent stimulatory effects on MHC antigen expression by a variety of cell types, including (in the case of IFN- γ) MCT cells (16, 41). The role of antigen-specific immune reactants is perhaps even more intriguing. α TBM Ab, for example, decrease class II MHC expression by tubular epithelial cells, an effect that is mediated at a transcriptional level (16). Such an effect may further diminish the importance of class II-restricted effector T cells *in vivo*.

We have demonstrated that M52 cells mediate both 3M-1-specific DTH and cytotoxic responses. The differentiation of cytotoxic and inflammatory functions of antigen-specific CD8⁺ T cells has not been previously studied in detail. As the pathology of many autoimmune lesions *in vivo* includes areas of both inflammation and target cell destruction, both functions may well be relevant to autoimmune injury (42-45). The interstitial lesions in α TBM disease are clearly comprised of areas reminiscent of a cutaneous DTH response, with a mononuclear cell infiltrate consisting of a heterogeneous population of T cells, B cells, plasma cells, natural killer cells, and macrophages (12). At times multinucleated giant cells are also apparent, and the lesions may have a granulomatous appearance. The *in vivo* evidence supporting the relevance of cytotoxicity consists of significant tubular cell drop-out, tubular dilatation, and subsequent scarring (12). Initial studies of M52 clones suggest these distinct functions are mediated by functionally distinct clones. The majority of our clones could transfer DTH responses and mediate comparatively inefficient cytotoxicity, whereas 1/17 clones is solely cytotoxic. These rela-

tive numbers of functionally defined clones may not quantitatively reflect the situation *in vivo*, as DTH-reactive clones may have a growth advantage *in vitro*. This analysis should be regarded instead as a qualitative assessment of functional attributes of the nephritogenic effector T cell repertoire.

M52 clones will be important reagents to better define mediators of cytotoxicity and inflammation in an autoimmune disease. Work by others, for example, has suggested that IFN- γ is an important mediator of DTH (46, 47). In preliminary studies examining cytokine expression by M52 clones, we have found that both M52.26 and the other M52 clones express IFN- γ , suggesting that other mediators are probably also important in this system (unpublished observations). Mechanisms of cytotoxicity by cytotoxic T lymphocytes (CTL) clones is an active area of research (48, 49). Exocytosis of granules containing such proteins as perforin, lymphotoxin, and serine esterases (48, 50, 51), have been implicated in effector cell-induced injury in other systems. Although there is still controversy regarding the relevance of these cytolytic proteins to *in vivo* cytotoxicity (48, 52, 53), recent studies have demonstrated expression of perforin at sites of autoimmune inflammation *in vivo* (54). It will be of interest to compare expression of cytolytic proteins in our functionally distinct CD8⁺ clones.

We were fortunate to find a peptide region of the 3M-1 molecule that is recognized by autoimmune effector T cells. Both P1 and P2 were selected from the open reading frame of a cDNA clone initially isolated from a λ gt11 library through screening with polyclonal α TBM antisera (11). Studies thus far support the hypothesis that P1 is a highly immunodominant region of the 3M-1 glycoprotein, since it is recognized by both helper and effector T clones. Curiously, it is also recognized by a monoclonal α TBM Ab, and is therefore an example of an antigenic epitope that can be recognized by TcR and antibody of the same antigenic specificity (11). Our studies do not eliminate the possibility of other immunogenic epitopes within 3M-1 which are important for the full expression of the autoimmune response. A precedent certainly exists for other minor immunogenic determinants in autoimmune disease (1, 3). The major immunogenic epitopes of 3M-1 may also be different in mice of different MHC haplotypes, or those who express TcR variable (V) genes that have been deleted in the SJL mouse (55). These issues are currently under investigation.

Autoimmune effector T cells have been characterized in several autoimmune disease models (42-45). In experimental allergic encephalomyelitis, the mononuclear infiltrate resembles a DTH reaction and the effector cells are CD4⁺, class II-restricted T cells with specificity for myelin basic protein. Multiple distinct encephalitogenic epitopes of myelin basic protein have been identified (1, 45). Several groups have demonstrated a striking bias in TcR α and β V gene usage by encephalitogenic CD4⁺ T cell clones (2, 56, 57), making it possible to use α TcR Ab recognizing specific V β families as a therapeutic modality (2, 57). Autoimmune T cells in collagen-induced arthritis in mice may also demonstrate a bias in V β usage (58). In several other autoimmune diseases, both CD4⁺ and CD8⁺ T cells are important in mediating organ damage. In murine experimental autoimmune thyroiditis, for example, adoptive transfer of thyroglobulin-specific CD4⁺ T cells results in thyroiditis, but CD8⁺ T cells are important in later stages of the disease (44). Both CD4⁺ and CD8⁺ T cells are required for the adoptive transfer of diabetes in the NOD mouse model (59, 60). The functional characteristics of these subsets have not been fully

defined, and it is not clear whether autoimmune T cells in NOD mice use limited TcR V genes.

In murine α TBM disease, adoptive transfer of either CD4⁺ or CD8⁺ antigen-specific T cells will result in interstitial nephritis, albeit with different kinetics. CD4⁺ T cells are not the ultimate effector cell but rather function to induce CD8⁺ effector cells in the recipient (6, 13). It is of interest that in a spontaneous model of autoimmune interstitial nephritis, the T cells responsible for adoptive transfer of disease are also CD8⁺, suggesting that the initial T cell injury to the kidney is frequently class I-restricted (33). As mononuclear cells infiltrate the kidney, and class II MHC expression is augmented by elaborated cytokines, CD4⁺ effector cells may also be recruited and function to sustain autoimmune injury. The chronic phases of autoimmune injury may also be maintained by immune responses to neoantigens expressed by damaged parenchymal cells. We are currently using the reagents described here to define more precisely the mediators of epithelial cell damage by autoimmune T cells, as well as those aspects of target cell biology altered by nonlytic interactions with T cells.

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