γδ T cells differentiate into a functional but nonproliferative state during a normal immune response


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ABSTRACT To obtain a homogeneous population of γδ T cells to investigate their role in an immune response, we have made a scid mouse doubly transgenic for rearranged γ and δ genes. The receptor (KN6) encoded by these genes is specific for the major histocompatibility complex class I protein encoded by the T22b gene. This mouse contains high levels of transgenic γδ T cells in the spleen and thymus and no other T lymphocytes. Immunization of these KN6-scid (H-2d, TLL-2) mice with 10^7 C57BL/6J (abbreviated B6) (H-2b, TLL-1) spleen cells resulted in proliferation and activation of the γδ T cells in spleen and clearing of the allogeneic B6 lymphocytes. Subsequently, the majority of activated cells died by apoptosis and the remaining cells were anergic with regard to proliferation. The anergic cells did not respond to restimulation by B6 spleen cells in vitro or in vivo, and addition of exogenous interleukin 2 failed to restore the response to B6 cells. Cytotoxicity, a property of KN6* cells during a primary stimulation, was no longer detectable in the proliferatively anergic cells. However, B6 spleen cells injected into mice primed 12 days previously were cleared with a much greater efficiency than on primary challenge and in an antigen-specific manner. We conclude that after exposure to antigen, γδ T cells rapidly proliferate into blasts and the majority of the blasts rapidly die, with the nonproliferating cells remaining in a highly active state for several weeks and able to initiate elimination of lymphoid cells bearing the TLL epitope.

Although many autoimmune diseases are caused by T cells (1), it has been difficult to follow single T cells through an immune response in vivo because of the inherent technical limitations of trying to study one T cell out of 10^9--10^10 T cells with different antigen specificities. To study the immune response of monoclonal T cells in vivo, free from the effects of other lymphocyte populations and also free from the effects of in vitro manipulations, we have made T-cell receptor (TCR)-transgenic scid mice. The scid mutation is an autosomal recessive gene defect that prevents normal T-and B-cell receptor gene rearrangement (2, 3). Introduction of an already rearranged TCR gene into the germ line of scid mice bypasses the gene defect and allows differentiation of functional T cells, all of the same specificity (4). A precedent for this approach has been established by Scott et al. (5).

We chose to produce a scid mouse carrying a γδ-TCR transgene, called KN6 after the anti-idiotypic antibody used to identify it, because the ligand of the KN6 receptor is known to recognize a class I major histocompatibility complex (MHC) antigen, the product of the T22b gene in the Tla locus (6--8). Introduction of the KN6 genes into C57/scid mice allowed us to study the fate of isolated γδ T-cell populations in response to peripheral stimulation with their T22b ligand.

MATERIALS AND METHODS

Mice. The KN6 founder male has been described (9). C57BL/6J (B6), BALB/c, B10.D2, B10.D2-H2d, and A.TL mice were obtained from The Jackson Laboratories. C57-scid mice and the KN6 γδ-scid mice were bred and maintained in the defined flora animal colony at the Ontario Cancer Institute (Toronto).

Antibodies and Cell Lines. The following monoclonal antibodies were used: biotinylated anti-KN6 (8D6) (10), biotinylated anti-C3 (GL3) (PharMingen), anti-CD3 (145-2C11) (obtained from the American Type Culture Collection (ATCC)), anti-H2b (SF1-1.1.1) (obtained from ATCC), anti-CD4-PE (YTS 191.1 from Coulter), and anti-CD8-PE (YTS 169.4 from Coulter). Mouse interleukin 2 (IL-2) cDNA-transfected X63-Ag8.653 cells were a generous gift of F. Melchers (11). All cell lines were maintained in RPMI 1640 medium + 10% fetal calf serum at 37°C in an atmosphere of 5% CO2.

Cell Preparations. Mice were sacrificed by cervical dislocation and single spleen cell suspensions were made by standard techniques (12).

Immunofluorescence and Flow Cytometry. Cells (10^9) were allowed to react with either 50 µl of culture supernatant or 1 µg of purified primary antibody in Dulbecco's phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.2% sodium azide on ice for 25 min. They were washed twice and subsequently counterstained with either streptavidin-phycocerythrin (Caltag, South San Francisco, CA) or fluorescein isothiocyanate (FITC)-conjugated goat antibodies to mouse immunoglobulin (Cedarlane Laboratories) as the secondary reagent.

Determination of KN6* Cell Numbers. Spleen cell suspensions were stained with biotinylated antibody 5C10 and streptavidin-phycocerythrin, and the number of KN6* cells was calculated from the percentage of 5C10* cells and the total cell number. Samples were analyzed on an EPICS Profile (Coulter) or FACScan (Becton Dickinson) flow cytometer.

DNA Fragmentation Assay. DNA was extracted from spleen cell suspensions depleted of erythrocytes and analyzed as previously described (13).

Proliferation Assay. The number of experimental cells included in each culture well was adjusted to ensure that the number of KN6* cells was the same as for controls. From each group, 5 × 10^4 cells were incubated with and without 5 × 10^3 irradiated [2000 rads (1 rad = 0.01 Gy)] B6 stimulator cells.

Abbreviations: TCR, T-cell receptor; B6, C57BL/6J; IL-2, interleukin 2; FITC, fluorescein isothiocyanate; MLR, mixed lymphocyte reaction; NK cell, natural killer cell.

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cells in 0.2 ml of 10% fetal calf serum, 50 μM 2-mercaptoethanol, and 10 mM Hepes in α-MEM (GIBCO) for 3 days. Twelve hours before termination of the cultures, 1 μCi (37 kBq) of [3H]thymidine was added to each well. Cultures were harvested onto fiber filter mats and radioactivities were measured in a scintillation counter. In some experiments, IL-2-containing supernatant was used in a 1:100 dilution, which previously had been found to support optimal proliferation of γδ T cells from KN6-scid mice. Results are reported as the average of four replicate wells and the error is the standard deviation. Net stimulation is the result after subtraction of the cpm of [3H]thymidine incorporated by B6 stimulator cells alone.

**Cytotoxicity Assay.** The tumor target P815 (H2b) was used in an antibody-redirected chromium release assay (14). Unless otherwise indicated, cells from spleen suspensions were used directly in the cytotoxicity assay. For the *in vitro* activation of cytotoxic cells, spleen cells were stimulated in a mixed lymphocyte reaction (MLR) for 4 days. At the end of this period, viable cells were isolated from the cultures by centrifugation over Lympholyte-M (Cedarlane Laboratories). The number of KN6+ γδ T cells in each reaction was about the same in all the groups. Specific release (p) was calculated as (experimental release – spontaneous release)/ (total release – spontaneous release) and reexpressed as cytotoxic activity, 100 × ln(1 − p) (15). Spontaneous release was always less than 10%. Five wells at each effector-to-target ratio were assayed, and the results are expressed as the mean ± SD.

**Labeling of Donor Cells with FITC.** In some experiments, persistence of donor cells was determined by directly labeling cells before injection into recipient mice, as has been previously described (16).

**RESULTS**

**Production and Characterization of KN6-scid Mice.** The KN6-scid mouse was produced by breeding a KN6 male to a C.B-17-scid female reconstituted with C.B-17 bone marrow. Offspring carrying the KN6 transgene were identified by PCR analysis of tail DNA, using primers specific for Vγ4 and Jγ1 as described elsewhere (17). These mice underwent a second round of breeding to scid animals, and offspring homozygous for the scid mutation were identified by the absence of serum immunoglobulin. Mice that were both KN6+ and immunoglobulin-negative were intercrossed through three more generations to produce a scid line homozygous for the KN6 transgene.

The KN6-scid mice are H-2^d/d as determined by flow cytometry using anti-H-2 antibodies. The thymus of a KN6-scid mouse is only 10% of normal size; 90% of KN6-scid thymocytes have the phenotype CD3^+^, CD4^−^, CD8^−^, a majority of KN6-scid splenocytes also have the γδ phenotype. The abundance of KN6+ cells in the spleens of KN6-scid mice is in contrast to the lack of αβ T cells in the periphery of αβ-TCR transgenic mice of C.B-17-scid background (5) or the lack of γδ T cells in the periphery of C.B-17-scid mice transgenic for the Vγ5/Vδ1 receptor from an epidermal γδ T-cell clone (18). There are no B cells and no CD4^+^ or CD8^+^ cells detectable in adult KN6-scid mice. There are low levels of KN6+ cells in the skin and intestine.

**KN6+ Cells Proliferate in Response to Antigen.** To examine the fate of antigen-stimulated γδ T lymphocytes, KN6-scid mice received an intravenous injection of 10^7 B6 spleen cells. KN6+ cells rapidly proliferated in response to B6 stimulation (Fig. 1 Left) and developed blast morphology (Fig. 1 Right). By day 4, the number of splenic KN6+ T cells had increased 4.5-fold compared with those in normal KN6-scid mice. Three observations indicate that this increase in the γδ T-cell number results from proliferation of cells in response to antigen in situ in the spleen rather than trapping of circulating cells by antigen. First, the blast morphology of the γδ cells on day 4 indicates that they have been activated by antigenic stimulation. Second, the percentage of KN6+ γδ T Cells in the blood on day 2 is nearly the same as on day 0 (Fig. 1 Left). Previous work has established that by 24 hr after injection of antigen, antigen-specific T cells are trapped at antigen sites (19); the constancy of γδ T cells in blood indicates that relatively few have been trapped by antigen in tissues. Third, when spleen cells from KN6-scid mice are placed in culture without any antigenic stimulation 2 days after injection of B6 spleen cells, their spontaneous proliferation is at least 2-fold greater than spleen cells from normal KN6-scid mice as measured by thymidine uptake (data not shown).

**KN6+ Blasts Undergo Apoptosis.** Spent and Miller suggested that activated T cells may migrate to the gut, where they are excreted or die (20); these factors may account for the decrease in γδ T cells after day 4. Although we have not evaluated possible excretion through the gut, we have observed substantial apoptotic cell death in the spleen cells of KN6-scid mice 4 days after stimulation *in vivo* with B6 spleen cells (Fig. 2); similar results have been obtained in four independent experiments. The percentage of B6 cells remain-
spleen cells did not proliferate in vivo in response to a second challenge with $10^7$ B6 spleen cells (Fig. 1), implying that day 12 cells were also unresponsive in vivo. Since addition of IL-2 reverses the anergy observed in αβ $\Gamma$ clones stimulated with antigen alone (21), we tested the ability of IL-2 to restore the responsiveness of γδ T cells from antigen-stimulated KN6-scid mice. Addition of IL-2 to MLR cultures had no effect on proliferation except for cells taken 19 days after immunization, when IL-2 restored the response to 50% of the control response (Table 1); the recovery at that time may be due to the emergence of newly produced γδ T cells from the thymus. Thymocytes examined 14 days after injection of B6 spleen cells show normal reactivity to B6 spleen cells (Table 1), indicating that the proliferative anergy occurs only in the peripheral immune system.

Anti-CD3 antibodies and Con A both activated normal γδ T cells from KN6-scid mice but had no effect on γδ T cells from KN6-scid mice exposed previously to B6 cells (Table 1). This observation supports the hypothesis of an intrinsic defect in the ability of in vivo stimulated KN6+ cells to make subsequent proliferative responses to antigen. To determine whether the hyporesponsiveness of the stimulated cells was due to a suppressive mechanism, previously immunized KN6-scid γδ T cells were tested for their ability to suppress various MLR responses. Hyporesponsive (day 12) cells had no effect when added to an unrelated MLR response mixture, BALB/c (H-2b) anti-B6 (H-2b). Addition of a 2-fold excess of hyporesponsive cells to normal KN6-scid spleen cells produced a 50% suppression of their ability to respond to B6 (data not shown). This modest suppression is likely a blocking effect of the hyporesponsive cells which can bind B6 antigen but not respond. The conclusion from these experiments is that hyporesponsiveness is an intrinsic property of antigen-stimulated γδ T cells.

A decrease in TCR density has been observed in some systems of peripheral tolerance (22, 23) but not in others (24). In our experiments the TCR densities of responsive and nonresponsive KN6+ cells were identical, and there were no excess CD3+ or Thy 1.2+ cells that might represent γδ T cells with their TCR down-regulated.

Stimulation of γδ T cells from normal KN6-scid mice with B6 spleen cells in vitro for 4 days demonstrated high cytotoxicity against P815 mastocytoma cells in an antibody-redirected cytotoxicity assay (14) (Table 2). In contrast, the same number of KN6+ cells taken from animals immunized 2 weeks earlier with B6 spleen cells did not develop cytotoxicity after a similar period of in vitro stimulation. Moreover, these cells were not constitutively cytotoxic nor could they be stimulated in vivo to exhibit cytotoxicity (Table 2). We conclude that, in addition to developing proliferative anergy, γδ T cells also lose their cytotoxic ability during an immune response.

Primed γδ T Cells Mediate Rejection of Allogeneic Cells in an Antigen-Specific Manner. We next examined the fate of the B6 donor cells in stimulated mice. If the KN6+ γδ T cells in immunized mice are functionally anergic, the response to a second challenge should be similar to the response of C.B-17-scid mice, which lack any detectable T cells. Four days after primary injection of $10^7$ B6 spleen cells into KN6-scid mice, the total number of persisting allogeneic B6 cells was $\frac{1}{2}$ of that in similarly challenged C.B-17-scid mice (Fig. 3); donor B6 cells were undetectable in KN6-scid recipients 7 days after their injection. However, a secondary challenge with B6 spleen cells of KN6-scid mice, 14 days after they were initially stimulated with B6 cells, was rejected much more strongly than the primary challenge (Fig. 3). B6 spleen cells could barely be detected in mice 4 days after a secondary challenge.

This rapid clearance should be antigen specific if it is mediated by previously stimulated KN6+ γδ T cells. After-
natively, this elimination may occur nonspecifically through previously activated natural killer (NK) cells or macrophages. Our experiments indicate that the rejection of allelo-
genetic lymphocytes by KN6-scid mice is antigen specific. Like BALB/c spleen cells, spleen cells from B10.D2 (H2d, TLb), B10.D2-H2dnull, and A.TL (H2K/D, TLβ) mice do not stimulate KN6+ cells (Table 1). Upon injection into KN6-scid mice previously injected with B6 spleen cells, none of these spleen cells are rejected (Fig. 3).

The mechanism of the accelerated clearance of B6 cells on secondary challenge is not entirely clear. We suggest that, while KN6+ γδ T cells cannot proliferate in response to secondary antigenic stimulation, they can release cytokines in response to such stimulation and that these cytokines activate cytotoxic mechanisms, perhaps NK cells or macrophages, which eliminate allelogenic lymphocytes. In support of the critical role of the previously stimulated KN6+ γδ T cells in this accelerated clearance, we have found that C.B-17-scid mice adoptively transferred with antigen-stimulated KN6+ γδ T cells 11 days after primary stimulation acquire the ability to clear a challenge by B6 spleen cells (Fig. 3).

**DISCUSSION**

Our experiments illustrate the utility of using TCR-transgenic scid mice for tracking the fate of specific T cells during an immune response in vivo. We made three conclusions from our studies: First, γδ T cells can proliferate in response to antigen, reaching a peak 4 days later. Second, after the peak response, many of the activated cells undergo programmed cell death. Third, the remaining cells are anergic with respect to proliferation and cytotoxicity but can still mediate antigen-specific resistance to a second challenge with allogeic lymphocytes; this role in rejecting allelogenic cells is a previously unrecognized function of γδ T cells.

Some of our results parallel those of three recent studies on the immune response of mature αβ T cells. Initial stimulation by antigen followed by long-term proliferative unresponsiveness has been seen in V88 CD4+- cells in response to the superantigen staphylococcal enterotoxin B (SEB) (24), with V88 CD4+ cells in response to MLS-1α (25), and with transgenic H-Y/Kb-specific CD8+ cells proliferating in B6 nude mice (22). Apoptosis has been reported only in the SEB system, although it may occur in the other systems as well. It is not clear if this sequence of events is a general property of activated T cells, since one of the systems involves the response of CD4+ T cells to superantigens and the other involves the response of CD8+ T cells to a tissue antigen.

The significance of programmed cell death of γδ T cells after activation is unclear. A possible explanation of the need for programmed cell death in an immune response is that after elimination of antigen as a result of a successful immune response it serves as a mechanism to rid the body of unnecessary immune cells via a noninflammatory pathway. Apop-
tosis may also provide a mechanism to avoid the develop-

**Table 1. Proliferative response of KN6+ γδ T cells after antigenic stimulation in vivo**

<table>
<thead>
<tr>
<th>Days after antigen injection source</th>
<th>γδ T-cell IL-2*</th>
<th>Proliferative response in vitro to various stimuli,† cm^3 × 10^−3 of [3H]thymidine incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None‡</td>
<td>B6 spleen</td>
</tr>
<tr>
<td>Control</td>
<td>1.9 ± 0.5</td>
<td>29.9 ± 6.6</td>
</tr>
<tr>
<td>Control</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 1.3</td>
</tr>
<tr>
<td>4 Spleen</td>
<td>1.1 ± 1.2</td>
<td>44.3 ± 3.2</td>
</tr>
<tr>
<td>12 Spleen</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 1.3</td>
</tr>
<tr>
<td>12 Spleen</td>
<td>1.6 ± 0.5</td>
<td>2.4 ± 1.6</td>
</tr>
<tr>
<td>12 Spleen</td>
<td>0.8 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>12 Spleen</td>
<td>0.8 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>12 Spleen</td>
<td>2.3 ± 0.6</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>19 Spleen</td>
<td>2.2 ± 0.6</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>14 Spleen</td>
<td>2.0 ± 0.6</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>14 Thymus</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>14 Thymus</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

ND, not done.

*Supernatant from X63Ag8-653 cells at 1:100 dilution. Control experiments showed that this concentration provided maximum proliferation of KN6+ γδ T cells in an MLR response to B6 stimulators.

†The MLR response of KN6+ γδ T cells is antigen specific.

‡Incorporation into KN6+ γδ T cells incubated for 4 days in culture without any antigenic or mitogenic stimulation.

§Net incorporation is the difference in incorporation between the B6-stimulated KN6+ γδ T cells and the nonstimulated cells.

**Table 2. Cytotoxic activity of KN6+ γδ T cells**

<table>
<thead>
<tr>
<th>Source of effector cells</th>
<th>Stimulating cells in MLR</th>
<th>Time in MLR,§ days</th>
<th>Cytotoxicity‡ at effector-to-target ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>60:1</td>
</tr>
<tr>
<td>KN6-scid spleen</td>
<td>None</td>
<td>0</td>
<td>4.0 ± 3.6</td>
</tr>
<tr>
<td>KN6-scid spleen</td>
<td>B6</td>
<td>4</td>
<td>99 ± 5.2</td>
</tr>
<tr>
<td>Day 14 after primary stimulation‡</td>
<td>None</td>
<td>0</td>
<td>2.5 ± 1.8</td>
</tr>
<tr>
<td>Day 14 after primary stimulation‡</td>
<td>B6</td>
<td>4</td>
<td>6.0 ± 1.4</td>
</tr>
<tr>
<td>Day 14 after primary stimulation plus 3 days after secondary stimulation§</td>
<td>None</td>
<td>0</td>
<td>3.0 ± 0.7</td>
</tr>
</tbody>
</table>

*In some experiments cells were cultured in MLR for 4 days prior to testing for cytotoxicity.

†Cytotoxic activity was determined by using an antibody redirected assay with P815 targets.

‡Spleen cells were harvested from KN6-scid mice 14 days after a primary immunization with 1 × 10^7 B6 spleen cells.

§KN6-scid mice were injected twice, day 0 and day 14, with B6 spleen cells. Spleen cells from the injected mice were harvested 3 days after the second injection of B6 cells.
ment of potentially neoplastic mutations in rapidly dividing T lymphocytes. While the mechanism of programmed cell death is also unknown for any T cells, IL-2 may play an important role. Apoptosis occurs in factor-dependent cell lines as a result of withdrawal of IL-2 (26); in other systems, apoptosis may result from high local levels of IL-2 (27).

Unresponsive aB T cells arise after superantigen stimulation and appear to be functionally anergic, although Bandeira et al. (28) demonstrated that anergic Vδβ cells can support immunoglobulin production and mediate resistance to a second challenge with Mls-1b-bearing cells. This observation is consistent with our finding that antigen-stimulated y8 T cells cannot proliferate but remain functional in their ability to eliminate allogeneic cells. In the studies on superantigen stimulation of aB T cells, it is difficult to evaluate the function of the unresponsive cells because of the heterogeneity of the population of responding T cells. Analysis of KN6-SCID mice has the distinct advantage that all of the T cells are identical and appear to respond simultaneously and identically to an antigenic stimulus. This homogeneity and synchrony made it easy to detect the nonproliferative population late in the immune response.

It is unclear how long y8 T cells remain in a nonproliferative state after antigen stimulation. The superantigen-induced anergic state in aB T cells is reversible and requires the absence of antigen (29, 30). The nonproliferative state of y8 T cells may be similarly reversible. Alternatively, the cells may die some time after stimulation in a manner similar to plasma cells produced after antigen activation of B cells.

The role of the functional, but nonproliferative, y8 T cells late in an immune response remains unclear. We favor the hypothesis that they are terminally differentiated long-lived lymphocytes involved in clearing antigen remaining from the initial challenge. The fate of activated aB T cells is different. Activated cytotoxic aB T cells continue to divide (31), and memory generation depends on increased numbers of other population of specific cytotoxic precursor cells (32). Memory helper aB T cells proliferate much more rapidly in response to antigenic stimulation than previously unstimulated cells (32) and are thought to arise through antigen-driven differentiation of virgin CD4+ cells. Previously activated KN6+ y8 T cells do not increase in number compared with unstimulated cells and, in contrast to memory aB T cells, lose their ability to proliferate in response to antigen. Thus, our experiments indicate either that y8 T cells mature differently than aB T cells during the course of an immune response or that a similar differentiated state for aB T cells has not yet been identified.

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