10^{-10} M (*12*), but, being derived from nonmutated germ-line immunoglobulin genes (*13*), they are predominantly of low affinity (*12*) and are nonpathogenic. Alternatively, when soluble self antigens are expressed at subthreshold concentrations, the failure to induce tolerance in high-affinity self-reactive B cells creates the potential for production of pathogenic autoantibodies of high affinity should T cell tolerance be bypassed (*7, 14*). Although most self antigens are present at amounts above this threshold, such a situation may explain why it has been relatively easy to raise high-affinity autoantibodies against certain autologous (*15*) or transgene-encoded (*16*) antigens in experimental models. Moreover, the nominal threshold of self antigen required for inducing B cell tolerance within the high-affinity component of the B cell repertoire may not be absolute but may be influenced by variables such as the tissue site of expression and the valency of individual antigens. Collectively, these findings suggest that the selective failure of B cell tolerance to particular self antigens may provide one route toward the development of clinical autoimmune disease, particularly under conditions where B cells are chronically stimulated either nonspecifically or by foreign antigens that cross-react or form complexes with self antigens (*14*).

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Peripheral Selection of the T Cell Repertoire

BENEDITA ROCHA AND HARALD VON BOEHMER

T lymphocytes undergo selection events not only in the thymus, but also after they leave the thymus and reside in the periphery. Peripheral selection was found to be dependent on T cell receptor (TCR)–ligand interactions but to differ from thymic selection with regard to specificity and mechanism. Unlike thymic selection, peripheral selection required binding of antigen to the TCR, and it induced expansion of T cell clones. Tolerance to self antigens that are restricted to the periphery occurred through the elimination of self-reactive T cells and by the clonal anergy, which was associated with down-regulation of the αβ TCR and CD8.

DEVELOPMENT OF T CELLS TAKES place in the thymus, where the TCR repertoire is generated by random gene rearrangement and pairing of TCR α and β chains. Potentially autoaggressive thymocytes are removed from this repertoire by "negative selection" (*1, 2*). Maturation of thymocytes requires "positive selection," which involves binding of the TCR to major histocompatibility complex (MHC) molecules (*3*): TCR interactions with class I MHC molecules direct $CD4^+$ $CD8^+$ thymocytes into the $CD8^+$ lineage, whereas interactions with class II MHC molecules result in maturation into $CD4^+$ cells (*4–7*).

Thymic selection may not be the sole mechanism by which the T cell repertoire is formed. Selection of T cell specificities may also occur in the periphery, to which the expression of certain self antigens may be limited. Tolerance to these antigens would require an extrathymic mechanism (*8*). In addition, peripheral selection events may expand or "suppress" certain clones. These selection events may be of particular importance in the normal adult mouse, in which the maintenance of the peripheral T cell compartment is largely independent of thymic output and the majority of T cells are generated by peripheral expansion (*9–14*): once the peripheral pool of T cells is seeded from the thymus early after birth, it may be sustained throughout life independently of further cell export from the thymus (*14*). Extrathymic expansion of T cells is not necessarily a response to exogenous antigens because it does not require intentional antigenic stimulation and it occurs in germ-free mice (*12, 13*).

To determine whether TCR-ligand interactions are required for peripheral expansion, we analyzed the in vivo expansion of αβ TCR transgenic T cells with receptors of known specificity. The genes for the α and β chains of a TCR that recognizes the male antigen HY when presented by the H-$2D^b$ class I MHC molecule were introduced into mice, which were then backcrossed to the C57Bl/6 (B6) background. In these mice, endogenous V_β gene segments do not rearrange, and the transgenic β chain is expressed on all T cells (*2*). Not all cells express the α transgene (*3*). Because specificity for HY requires the transgenic α and β chains (α_T and β_T) as well as CD8 molecules (*15*), male antigen–specific cells are $CD8^+$ with high levels of expression of α_T ($CD8^+\alpha_T$ cells). Because positive selection occurs in the absence of the HY antigen, $CD8^+\alpha_T$

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cells are generated in the thymus of female H-2D^b B6 transgenic mice and migrate to the periphery, where they represent approximately one-half of all CD8$^+$ cells. The rest of the CD8$^+$ cells, as well as the vast majority of CD4$^+$ T cells, express endogenous α chains (α_E) paired with β_T (CD8$^+\alpha_E$ cells and CD4$^+\alpha_E$ cells); these cells, in addition to CD4$^-$CD8$^-\alpha_T$ cells, which constitute about 5% of T cells, do not respond to male antigen in vitro (*16*).

We transferred female transgenic cells into female and male nude mice (*17*). Transgenic donor cells can be identified in the recipients by the expression of β_T, which can be detected by the monoclonal antibody (MAb) F23.2 (Fig. 1). This MAb binds the V_β8.2 chain, which is absent from host B6 nude mice (*18*). Male antigen–specific CD8$^+$ T cells can be identified by the MAb T3.70, which recognizes α_T (*3*). Therefore, the fate of male antigen–specific CD8$^+\alpha_T$, CD4$^+\alpha_E$, and CD8$^+\alpha_E$ cells can be followed in recipient nude mice after adoptive transfer.

CD4$^+\alpha_E$ and CD8$^+\alpha_E$ cells from transgenic mice expanded exponentially after transfer into female or male nude recipients (Fig. 2). The total number of donor transgenic α_E cells recovered from adoptive hosts at different times after transfer and the kinetics of expansion of CD4$^+\alpha_E$ and CD8$^+\alpha_E$ cells were similar in female and male nude mice (Fig. 2). T cell proliferation in these mice was similar to that in nude mice injected with normal female B6 spleen cells (*12*). At early times after transfer, most donor T cells were CD4$^+$ (*12*) (Fig. 2). CD8$^+$ cells, which expand more slowly, accumulated later. CD4$^+\alpha_E$ to CD8$^+\alpha_E$ ratios similar to those observed in the donor cell inoculum were observed in recipient nude mice 2 weeks after injection. After that time, the total number of donor cells remained constant (*12*) (Fig. 2). In contrast to $\beta_T\alpha_E$ cells, CD8$^+\alpha_T$ male antigen–specific cells expanded differently in male and female nude mice. In male nude mice injected with transgenic cells, CD8$^+\alpha_T$ cells expanded quickly (Fig. 2). Greater than 80% of donor T cells in male nude mice were CD8$^+\alpha_T$ at day 5 after transfer. The absolute number of T cells recovered in the spleen of male mice is four times that found in female mice injected with the same cells, and the number of CD8$^+\alpha_T$ cells is 50 times that found in females (Fig. 2). Similar differences were observed in lymph nodes. These results demonstrate that CD8$^+\alpha_T$ mature T cells are able to recognize and proliferate in response to male antigen in vivo as they do in vitro (*3*).

Male antigen–specific cells did not expand after transfer into female nude mice: the absolute numbers of CD8$^+\alpha_T$ cells did not vary with time after injection (Fig. 2) and corresponded to the fraction of donor T cells expected to home to peripheral lymphoid organs after intravenous injection (Fig. 2) (*19*). Because of the preferential expansion of T cells with other TCR specificities, CD8$^+\alpha_T$ cells were diluted in the host, so that 1 month after transfer they

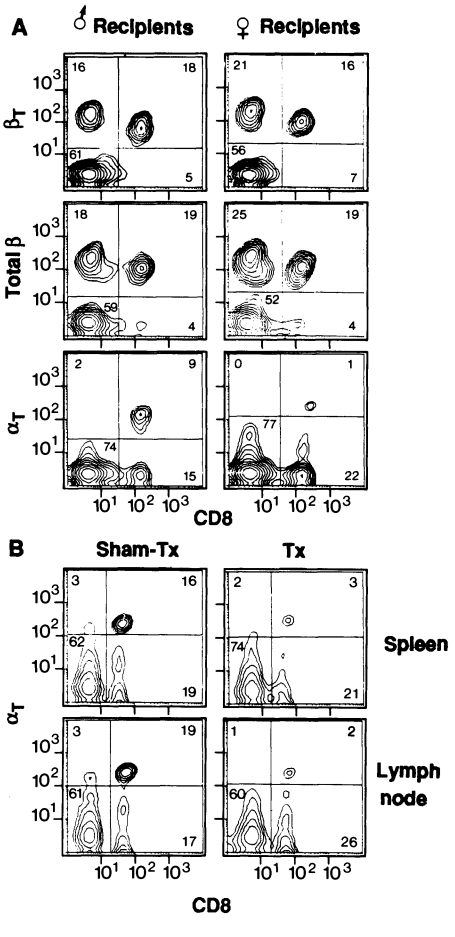

Fig. 1. Frequency of CD8$^+\alpha_T$ cells after adoptive transfer or adult thymectomy. (**A**) Distribution of female transgenic T cells in male (left panel) or female (right panel) nude mice 1 month after injection. Eight-week-old female or male B6 nude mice were injected intravenously with 10^7 syngeneic spleen T cells from B6 transgenic mice and then killed 1 month later. The percentages of CD4$^+$ and CD8$^+$ T cells in donor transgenic cell populations were 50% and 42%, respectively. Among CD8$^+$ cells, 53.2% were α_T, and among CD4$^+$ cells, 3.7% were α_T. Spleen cell suspensions from a pool of three recipient mice per experimental group were depleted of B cells by magnetic sorting with Dynabeads (Dynal A.S., Oslo, Norway) coated with antibodies to immunoglobulin. Cells were double-stained (*18*) with fluorescein isothiocyanate (FITC)–conjugated MAb HO22 to CD8 (*24*) and biotinylated MAb to the TCR together with streptavidin-phycoerythrin. MAbs to the TCR were F23.2, which recognizes β_T (*25*); T3.70, which recognizes α_T (*3*); and H57-597, which recognizes all β chains (*26*). Two-color fluorescence was analyzed as described (*18*) with a FACScan (Becton Dickinson). Results represent one out of four experiments. (**B**) Effects of adult thymectomy in transgenic female mice. The percentage of male antigen–specific cells recovered from the spleen and a pool of peripheral and mesenteric lymph nodes of adult thymectomized (Tx) or sham-thymectomized (Sham-TX) female transgenic mice, 1 month after surgery, is shown. Eight-week-old female transgenic mice were sham-thymectomized or thymectomized under light (ether) anesthesia (*11*). Transgenic cell populations were analyzed as described in (A). Results are from one of three similar experiments.

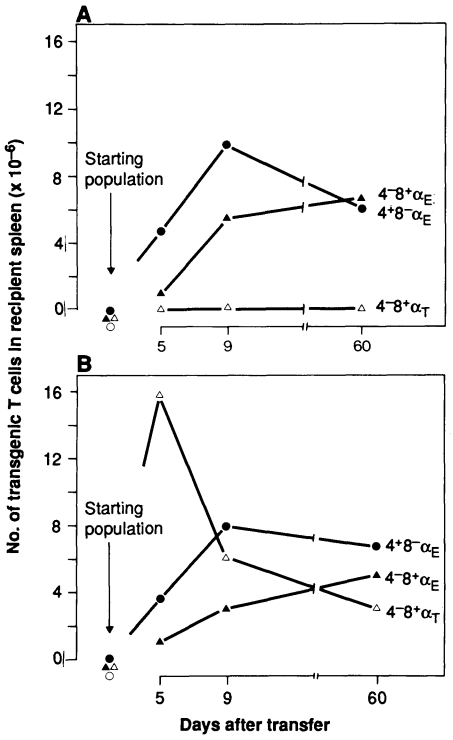

Fig. 2. Expansion of transgenic T cells after transfer into female and male nude mice. Results represent the absolute number of transgenic lymphocytes recovered in the spleen of recipient female (**A**) and male (**B**) nude mice at different times after injection. Numbers represent the mean of two mice for each experimental group. B6 nude mice were injected simultaneously with 10^7 T cells from female B6 transgenic mice. Recipients were killed on different days after transfer. For determination of the total number of cells recovered in each organ, a standard procedure was used to prepare exhaustive cell suspensions from the spleen, and three independent samples from each suspension were counted before washing (*12*). Cell suspensions were incubated with FITC-labeled MAb to Thy-1 (*12*) and the total number of Thy-1$^+$ cells present in recipient organs was evaluated from the percentage of Thy-1$^+$ cells and the number of cells recovered in the spleen of each recipient. Cell suspensions were then depleted of B cells, and Thy-1$^+$ donor cells were enumerated within the Thy-1$^+$ cell population by analyzing the co-expression of CD4 [CD4$^+$ cells were identified with FITC-conjugated MAb GK1.5 (*27*)] or CD8 molecules with the transgenic TCR chains. Significant numbers of CD4$^+\alpha_T$ cells were not detected in recipient organs. The number of donor cells homing to the spleen after in vivo transfer was considered to be 20% of the injected inoculum (*19*). Similar results were obtained in four independent experiments.

represented only 5% of the $CD8^+$ cells in female nude mice (Fig. 1A). Thus, peripheral expansion of male antigen–specific cells required the interaction of the TCR with its antigen. Because the HY antigen is not encountered in female mice, HY-specific T cells do not expand there, whereas α_E T cells, which express other TCR specificities, do expand in the same recipients; these cells may proliferate in response to environmental antigens, or as a result of cell interactions with other components of the immune system.

Unlike in female nude hosts, $CD8^+\alpha_T$ cells were prevalent in female donor transgenic mice (Fig. 1B). This abundance depended on a continuous export of cells from the thymus, as was demonstrated by adult thymectomy (Fig. 1B). In sham-thymectomized female transgenic mice, $CD8^+\alpha_T$ cells represented 46% and 53% of splenic and lymph node $CD8^+$ cells, respectively, whereas they represented only 12% and 7% of splenic and lymph node $CD8^+$ T cells in thymectomized mice 4 weeks after surgery. The absolute number of $CD8^+\alpha_T$ cells recovered from these mice was only 10% of that recovered from sham-operated controls.

Thus, thymic and peripheral selection are different. In the thymus, the binding of a TCR to an MHC molecule in the absence of antigen results in maturation but not expansion of T cells. In contrast, peripheral selection involves expansion of T cells, the receptors of which bind to specific antigens. Thus, in our transgenic female B6 mice, the majority of single-positive thymocytes are $CD8^+\alpha_T$ cells (*3*). Because in the absence of the HY antigen, these cells cannot expand in the periphery (Fig. 2A), $\beta_T\alpha_E$ cells represent 75% of peripheral T cells in these mice (*3*) (Fig. 1B). Male antigen–specific cells present in secondary lymphoid organs of female transgenic mice are maintained by thymus output (Fig. 1B). Therefore, peripheral expansion does not simply reflect amplification of the TCR repertoire initially selected in the thymus but can induce considerable modifications in the frequency of initially selected clones.

We also analyzed the behavior of mature self-reactive $CD8^+$ T cells in male recipient nude mice, where they are continuously stimulated by HY antigen present in high amounts in tissues outside the thymus. Although $CD8^+\alpha_T$ cells expanded vigorously in male recipients during the first 5 days after transfer (Fig. 2), they caused no obvious pathological damage (*20*). The reasons for this are unclear. The differentiation of these cells into aggressor cells may require factors derived from $CD4^+CD8^-$ cells (*8*), or the action of male antigen–specific cells may be suppressed by other cells with endogenous TCR chains. Most (70%) $CD8^+\alpha_T$ T cells generated during the first 5 days after injection disappeared from peripheral lymphoid organs between day 5 and day 9 after transfer (Fig. 2B). After day 9 the decline was less rapid.

$CD8^+\alpha_T$ cells that were not eliminated from the peripheral lymphoid organs of male nude mice did not respond to antigenic stimulation by male cells in vitro (Table 1). Similarly, such cells did not proliferate in vivo after secondary transfer into male nude mice or into female nude mice that had been immunized with male cells. $CD8^+\alpha_T$ cells harvested from male nude mice 5 days after injection could respond to the HY antigen in vitro, whereas cells recovered at day 9 or day 20 could not (Table 1). This unresponsiveness could be correlated with the down-regulation of TCR and CD8 on the surface of $CD8^+\alpha_T$ cells. This process was progressive: the mean CD8 intensity was reduced by a factor of 2 at day 5 and by a factor of 3 after day 9 (Fig. 3A), and the TCR density on male antigen–specific cells also declined with time (Fig. 3A).

The down-regulation of TCR and CD8 at the surface of T cells was associated with the tolerance process because it was confined to the $CD8^+\alpha_T$ population: the density of CD8 and TCR on $CD8^+\alpha_T$ cells recovered from female recipient nude mice was identical to that of the donor $CD8^+\alpha_T$ cells. T cells expressing α_E had identical CD4, CD8, and TCR levels in male and female recipients (Fig. 3B). The expression of β_T was biphasic in $CD8^+$ T cells recovered from male recipients because they contained α_T and α_E

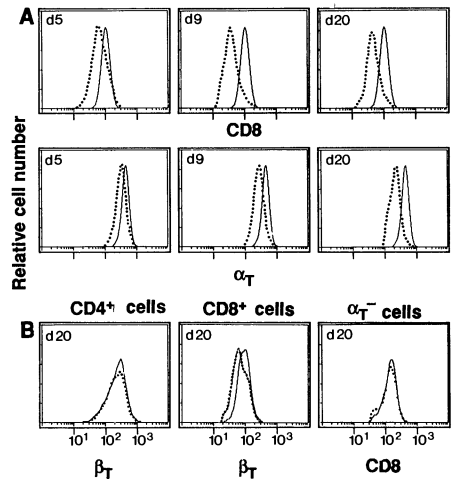

Fig. 3. Expression of TCR, CD4, and CD8 in transgenic cells after transfer into nude mice. (**A**) Density of expression of CD8 (upper panel) or α_T (lower panel) by male antigen–specific cells. Dotted lines, cells recovered from male nude recipients at different days (d) after transfer. Solid lines, cells recovered from female nude recipients that had the same CD8 or TCR expression as male antigen–specific donor cell populations. Similar results were obtained in seven independent experiments. Female or male nude mice were injected intravenously with 10^7 transgenic female T cells at days 5, 9, or 20 before they were killed. The percentages of $CD4^+$ and $CD8^+$ T cells in donor T cell populations were 49.7 ± 0.2% and 43.4 ± 1.8%, respectively. Among $CD8^+$ cells, 54 ± 2% were α_T, whereas among $CD4^+$ cells, 2.2 ± 1% were α_T. All mice were killed on the same day and all cell suspensions were labeled simultaneously with MAbs to CD8 and α_T. $CD8^+$ cells were gated for acquisition. (**B**) Expression of TCR and accessory molecules by transgenic α_E cells 1 month after transfer. Dotted lines, cells recovered from male nude mice; solid lines, cells recovered from female nude mice. The density of β_T at the surface of $CD4^+$ (left panel) and $CD8^+$ (middle panel) T cells is shown. $CD8^+$ T cells recovered from male mice contain both α_T and α_E cell populations (Fig. 1). (Right panel) Density of CD8 at the surface of $CD8^+\alpha_E$ cell populations.

Table 1. Responder cells (lymph node cells depleted of B cells and $CD4^+$ cells) (5×10^4) were cultured with 5×10^5 x-ray–treated (3000 R) spleen cells from male or female nude (nu/nu) mice in the presence or absence of exogenous interleukin-2 (IL-2). Responder cells were obtained from female transgenic mice or from nude male mice injected with female transgenic cells at days 5, 9, and 20 after cell transfer. The number of $CD8^+\alpha_T$ cells present in each culture was 2×10^4 in rows 1 and 4, 4×10^4 in row 2, and 3×10^4 in row 3. Values represent the mean [^{3}H]thymidine uptake (cpm) of triplicate cultures. SEM values were ≤10% of the means. Cells from female nude recipients, which contained less than 5×10^3 $CD8^+\alpha_T$ cells, always produced a significant male antigen–specific response in vitro. Similar results were obtained in eight independent experiments.

Responder cells	Spleen cells			
	nu/nu ♂ +IL-2	nu/nu ♀ +IL-2	nu/nu ♂	nu/nu ♀
♀ αβ $CD8^+$	150,000	4,800	37,400	600
♀ αβ $CD8^+$ from nu/nu ♂, day 5	74,500	35,200	17,500	5,000
♀ αβ $CD8^+$ from nu/nu ♂, day 9	42,300	46,000	1,000	1,700
♀ αβ $CD8^+$ from nu/nu ♂, day 20	7,400	5,800	600	700

populations (Fig. 3B). Therefore both α and β chains were down-regulated in $CD8^+\alpha_T$ but not in $CD8^+\alpha_E$ cells. This suggests that the induction of anergy in T cells (which is associated with down-regulation of the TCR and its coreceptors) probably requires a critical threshold of receptor occupancy, as has been described for mature B cells (*22*): Transgenic α_E cells, the expansion of which is also antigen-dependent, do not down-regulate their receptors (Fig. 3B).

Our results indicate that binding of the transgenic TCR to male antigen has different consequences in the thymus and periphery. Immature thymocytes die without cell division (*23*), whereas mature T cells proliferate vigorously at first but then either disappear or down-regulate their receptors and so become refractory to antigenic stimulation. The latter mechanism may be one of many by which peripheral T cells can be "silenced" and may be similar to that which has been described for mature B cells (*22*)—which can also down-regulate surface immunoglobulin expression and become unreactive. The differential behavior of thymocytes and mature T cells with regard to confrontation of self antigens lends support to the hypothesis of Ledenberg (*23*), which proposes different consequences of antigenic stimulation in immature versus mature lymphocytes. Nevertheless, our experiments also suggest that elimination of self-reactive clones can also occur in the periphery as a result of encounters between mature T cells and antigen. In contrast to the thymus, this cell elimination is not sudden, and its cause, as well as its mechanism, needs further investigation.

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14. At 3 weeks after birth, T cell function and numbers in peripheral lymphoid organs correspond to those in adult mice. This correspondence depends on extrathymic expansion because thymectomy interferes with this development only if performed before the second day after birth (*9*). Evaluation of cell production in the central and peripheral lymphoid organs of adult mice, into which T cell migration from the thymus is minimal (*11*), has also established that the majority of T cells are generated by postthymic expansion (*10*). The expansion potential of T cells is of similar magnitude to that of colony-forming units (*12*), which explains the relative independence of the peripheral T cell compartment from cell export from the thymus.
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Split Anergy in a $CD8^+$ T Cell: Receptor-Dependent Cytolysis in the Absence of Interleukin-2 Production

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Engagement of the antigen-specific receptor (TCR) of $CD4^+$ T lymphocytes without a second (costimulatory) signal prevents the subsequent production of interleukin-2 (IL-2) by these cells. Because IL-2 is a key immunoregulatory lymphokine and is also produced by a subset of $CD8^+$ T cells that are able to kill target cells, the effect of engaging the TCR of one such clone in the absence of costimulatory signals was examined. The capacity for TCR-dependent IL-2 production was lost, indicating comparable costimulator-dependent signaling requirements for IL-2 production in $CD4^+$ and $CD8^+$ T cells. However, TCR-mediated cytotoxicity was not impaired, implying that costimulation is required for only certain TCR-dependent effector functions.

THE ESTABLISHMENT OF A SELF-TOLERANT state among T cells is essential to the development of a useful immune system that responds to foreign antigens without undesirable self-reactivity. Defects in self-tolerance result in autoimmune disease, and knowledge about basic mechanisms of tolerance induction may prove beneficial for treatment or prevention of such diseases, as well as for efforts aimed at increasing the acceptance of histoincompatible tissue grafts. Two distinct mechanisms of T cell tolerance have been defined and studied at the clonal level: deletion, which is the result of programmed cell death and occurs predominantly during intrathymic maturation of T cells (*1*); and anergy, a state of viable T cells that is characterized by diminished or absent lymphokine secretion as a result of TCR engagement (*2*). Anergy can be induced in differentiated, mature $CD4^+$ type 1 (T_H1) T cell clones (*3*) by exposure to complexes of antigen and appropriate major histocompatibility complex (MHC) molecules in the absence of certain uncharacterized "costimulatory" signals on the antigen presenting cell (APC) (*4*). This anergy is most apparent as a lack of both IL-2 production and clonal proliferation after subsequent exposure to antigen and fully competent APCs. A subset of murine $CD8^+$ T cells can secrete IL-2, and previous studies of this population indicated that adherent cells provided antigen-unspecific, costimulatory signals that were essential for optimal proliferation and secretion of IL-2 in response to alloantigenic spleen cells (*5*). To explore whether this lack of response in the absence of costimulation might be associated with induction of a state of anergy, we isolated a class I ($H\text{-}2D^b$)–restricted, IL-2 producing, cytotoxic $CD8^+$ clone, termed GX1 (*6*), from the lymph node cells of a C57Bl/6 (B6) mouse that had been immunized with trypsin-digested chicken ovalbumin (T-OVA) (*7*).

When APCs are chemically fixed, they cannot stimulate IL-2 secretion or the proliferation of T_H1 $CD4^+$ T cells (*8, 9*). To determine if the $CD8^+$ clone GX1 had a similar response pattern, we exposed GX1

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