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Immunology

Organ injury associated with extrathymic induction of immune tolerance in doubly transgenic mice

(autoimmunity/alloreactivity)

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ABSTRACT **The developmental fate of autoreactive T cells encountering extrathymically expressed self-antigen was studied in a doubly transgenic mouse model system where pancreatic acinar cells expressed H-2L^d and T cells expressed an antigen receptor (2C TCR) specific for H-2L^d. Thymocytes bearing 2C TCR differentiated normally. They were positively selected without evidence of intrathymic clonal deletion. Survival of H-2L^d-bearing skin allografts was significantly prolonged in pancreatic H-2L^d singly and doubly transgenic mice, consistent with an *in vivo* state of T-cell tolerance. The mechanism of tolerance induction was determined and found to have two components. First, up to 80% of peripheral CD8$^+$2C TCR$^+$ T cells were eliminated. Second, those T cells which escaped elimination had a significantly reduced proliferative response to H-2L^d. Thus, autoreactive T cells can be made self-tolerant through interaction with self-antigen located extrathymically. This is accomplished by a reduction in the percentage of autoreactive T cells as well as by a reduction in the functional capacity of residual T cells. Surprisingly, although pancreatic lymphocytic infiltration and organ injury were absent in exocrine tissue of singly transgenic mice, it was present in doubly transgenic mice. This suggests that when the percentage of autoreactive T cells is high, tolerance induction can be associated with an inflammatory infiltrate in extrathymic tissue where self-antigen is presented.**

Transgenic animal model systems have been established to study extrathymic (peripheral) tolerance induction because they permit specific tissue localization of a novel antigen throughout T-cell ontogeny (1). Some investigators have reported the induction of T-cell unresponsiveness to peripherally expressed antigen (2–6), whereas others have found normal or near normal responsiveness (7–11). In those studies where functional tolerance was found, it was unclear whether autoreactive T cells were deleted or simply rendered functionally unresponsive, because it was not possible to track specific T cells *in vivo*.

To address this question, we established a doubly transgenic mouse model system that allows one to track T cells that possess a single antigen specificity during development in the presence of a peripherally located self-antigen. Doubly transgenic mice were derived by mating singly transgenic mice in which the self-antigen (H-2L^d) is expressed on pancreatic acinar cells to singly transgenic mice in which T cells express an antigen receptor (2C TCR) that is specific for H-2L^d (12). The fate of 2C TCR-bearing lymphocytes was determined by using an anti-clonotypic monoclonal antibody (13) and flow cytometric analysis. Thymocytes underwent positive selection without evidence of clonal deletion in doubly transgenic mice. An elimination of up to 80% of CD8$^+$2C TCR$^+$ peripheral T cells was found. This was also associated with a reduction in the functional responsiveness of residual T cells. In addition, spontaneous lymphocytic infiltrates and organ injury were observed in exocrine pancreas of doubly transgenic mice but not in singly transgenic mice. Thus, when the number of autoreactive T cells is high, the induction of tolerance to peripherally located self-antigen may be associated with lymphocytic infiltration and injury of the presenting tissue.

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EXPERIMENTAL PROCEDURES

Genomic DNA Constructs and Production of Transgenic Mice. The H-2L^d gene (LL) or a hybrid gene (EL), constructed by placing a 4.7-kilobase (kb) *Bam*HI L^d-gene fragment downstream of a 0.2-kb rat pancreatic elastase I promoter fragment (14, 15), was microinjected into F_2 zygotes. To make doubly transgenic animals, EL transgenic mice of the H-2^b haplotype were mated to 2C TCR transgenic mice of the same haplotype (12).

Assessment of Transgene Expression. Total RNA was isolated from mouse tissues (16), size-fractionated by agarose gel electrophoresis, transferred to a 0.45-μm nylon membrane (Micron Separations, Westboro, MA), and hybridized with a ^{32}P-end-labeled oligonucleotide probe specific for the α_2 domain of H-2L^d (5′-ACT-CAC-ACA-CTC-CAG-TGG-ATG-TAC-GGC-TGT-GAC-G-3′). Tissues from EL mice were frozen and 9-μm-thick sections were stained with a biotinylated anti-H-2L^d monoclonal antibody, 30-5-7 (17), followed by a streptavidin–horseradish peroxidase conjugate (Vector Laboratories) and diaminobenzidine (Sigma). Sections were counterstained with methyl green. Hematoxylin and eosin-stained sections of 10% formalin-fixed tissues were also examined. LL transgene expression was established by flow cytometric analysis of peripheral blood lymphocytes.

Flow Cytometric Analysis. Cells from 2C TCR mice were stained with a biotinylated anti-2C TCR monoclonal antibody called 1B2 (13). Secondary staining was performed with streptavidin–fluorescein isothiocyanate (Southern Biotechnology Associates, Birmingham, AL), streptavidin–phycoerythrin (Southern Biotechnology Associates), streptavidin–red 613 (GIBCO), directly conjugated anti-CD4 (GK1.5; PharMingen, San Diego), or directly conjugated anti-CD8 (53-6.7; PharMingen). Analysis was performed on a FACScan flow cytometer (Becton Dickinson).

Proliferation Assay. Splenic T cells were prepared by passing splenocytes over an anti-immunoglobin affinity column (Biotex Laboratories, Edmonton, AB, Canada). One hundred thousand splenic T cells or lymph node cells were mixed with 5×10^5 irradiated LL splenocytes. Cells from 2C TCR transgenic mice were cultured for 3 days and cells from nontransgenic mice were cultured for 4 days in 95% O_2/5% CO_2 at 37°C. Cells from transgenic or nontransgenic mice were then incubated with [^{3}H]thymidine (0.6 μCi per well;

Abbreviation: TCR, T-cell antigen receptor.

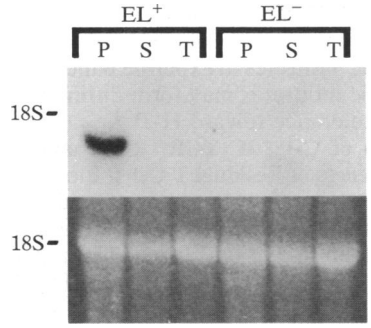

FIG. 1. Expression of the EL construct is pancreas-specific. Northern blot analysis was performed using total RNA (10 μg per lane) isolated from pancreas (P), spleen (S), and thymus (T) of EL transgenic (EL^+) and nontransgenic (EL^-) mice. Hybridization with an oligonucleotide specific for the α_2 domain of H-2L^d was performed as described in *Experimental Procedures* (*Upper*). Ethidium bromide staining shows comparable amounts of RNA in each lane (*Lower*). Position of 18S rRNA is indicated.

New England Nuclear; 1 μCi = 37 kBq) for an additional 6 or 16 hr, respectively. Mouse recombinant interleukin 21 (Genzyme) was used at a final concentration of 20 units/ml.

Skin Graft. Skin grafts were performed by using an adaptation of a previously described method (18). Tail skin from C57BL/6 and LL transgenic donors was grafted onto 7-week-old recipients. Grafts were checked daily for the first 4 weeks and periodically thereafter.

RESULTS

Generation of Doubly Transgenic Mice. Expression of the LL transgene and function of its product was established by flow cytometric analysis, proliferation assay, and crossing to 2C TCR transgenic mice (data not shown; ref. 12). Pancreas-specific expression of the EL transgene was demonstrated by Northern blot analysis (Fig. 1) and by immunohistochemical staining of sections from frozen pancreas and other tissues (data not shown). To demonstrate that H-2L^d expressed on pancreatic acinar cells was functional, anti-BALB/c alloreactive splenic T cells from C57BL/6 mice were adoptively transferred into EL transgenic mice. Two weeks after cell transfer, an intense pancreatic lymphocytic infiltrate associated with acinar cell destruction but preservation of islets and ducts was noted (data not shown). To permit tracking of 2C TCR-bearing T cells during development in the context of peripherally expressed H-2L^d, EL (EL^+2C^-) singly transgenic mice were crossed with 2C TCR (EL^-2C^+) singly transgenic mice.

Normal Differentiation of 2C TCR-Bearing Thymocytes. No difference was noted in flow cytometry profiles of thymocytes from doubly (EL^+2C^+) transgenic and 2C TCR (EL^-2C^+) transgenic littermates. 2C TCR-bearing cells were positively selected in doubly transgenic mice without evidence of clonal deletion (Fig. 2). Subset percentages (reported as mean ± SD) for 8-week-old EL^+2C^+ (n = 5) and EL^-2C^+ (n = 3) transgenic mice were as follows: $CD4^-CD8^-$, 31.2 ± 8.5 vs. 33.9 ± 5.2; $CD4^+CD8^+$, 30.0 ± 4.3 vs. 30.5 ± 6.7; $CD4^-CD8^+$, 32.0 ± 5.4 vs. 28.3 ± 2.0; $CD4^+CD8^-$, 6.8 ± 1.5 vs. 7.3 ± 1.0; $1B2^+CD8^+$, 59.2 ± 9.5 vs. 58.1 ± 1.0; $1B2^+CD8^-$, 29.6 ± 10.3 vs. 35.6 ± 4.1; $1B2^-CD8^+$, 4.7 ± 3.0 vs. 3.0 ± 1.9; and $1B2^-CD8^-$, 6.4 ± 2.7 vs. 3.3 ± 1.3. The relative proportion of thymocyte subsets was also similar at 16 weeks of age (data not shown). Thus, data from Northern blotting and flow cytometry are consistent with a lack of functional expression of H-2L^d in thymus from EL transgenic mice.

Pancreatic Lymphocytic Infiltrates with Acinar Cell Injury in Doubly Transgenic Mice but Not in Singly Transgenic Mice. To assess whether spontaneous autoimmunity against H-2L^d expressed on acinar cells occurred, pancreas from EL transgenic mice was analyzed by hematoxylin and eosin staining. EL and 2C TCR singly transgenic mice did not develop lymphocytic infiltration over a period of up to 6 months (zero of six and seven mice, respectively). In contrast, mild lymphocytic infiltrates were observed in pancreas from 33% (three of nine) of 8-week-old doubly transgenic mice (Fig. 3). Two to 4-week-old doubly transgenic mice did not have pancreatic infiltration (data not shown). More intense infiltrates were detected in 75% (six of eight) of 16-week-old doubly transgenic mice. Infiltrates were associated with acinar cell injury and loss. Pancreatic islets, ducts, and other

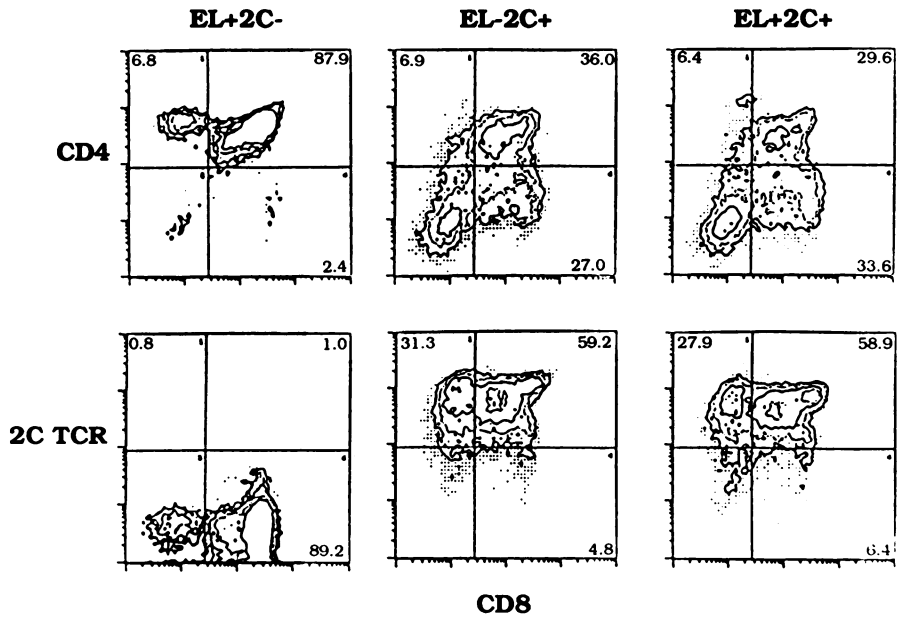

FIG. 2. 2C TCR-bearing thymocytes from doubly transgenic mice are positively selected without evidence of clonal deletion. Representative flow cytometric analyses of thymocytes from EL (EL^+2C^-), 2C TCR (EL^-2C^+), and doubly (EL^+2C^+) transgenic mice are shown. One million cells were stained and analyzed as described in *Experimental Procedures*. Percentage is indicated in each quadrant.

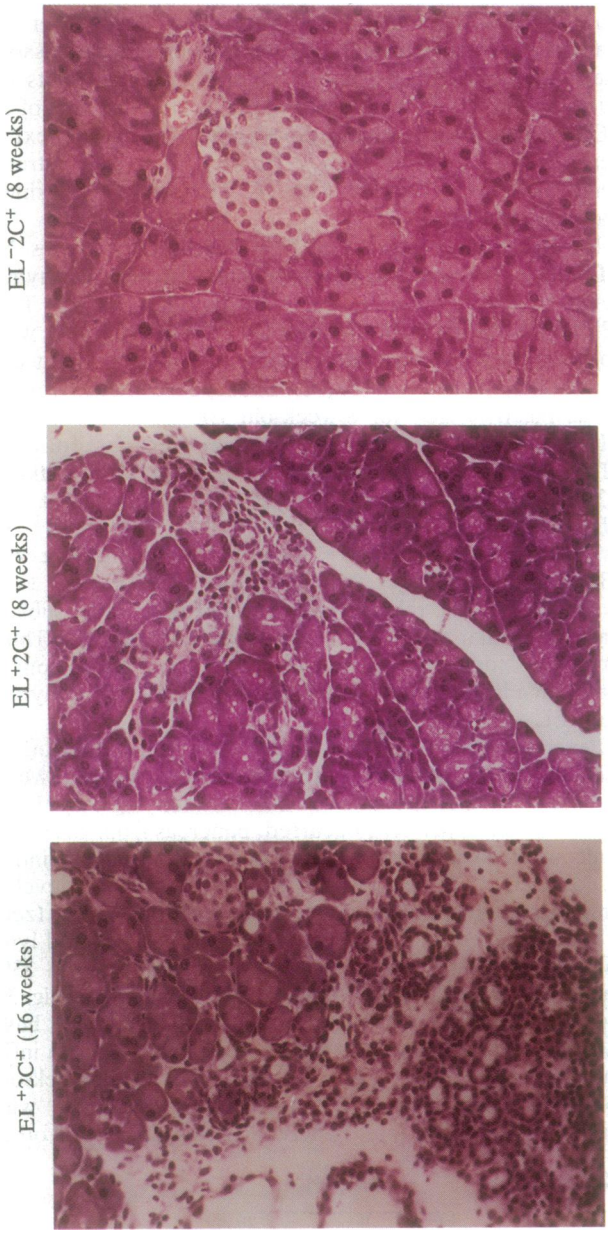

FIG. 3. Spontaneous pancreatic infiltrates are detectable in doubly transgenic (EL^+2C^+) mice but not in EL transgenic mice (data not shown) or 2C TCR (EL^-2C^+) transgenic mice. Hematoxylin and eosin-stained sections of pancreas from 8-week-old EL^-2C^+ and EL^+2C^+ transgenic mice (*Upper* and *Middle*; $n = 7$ and $n = 9$, respectively) and 16-week-old EL^+2C^+ transgenic mice (*Lower*; $n = 8$) are shown. (×160.)

tissues (heart, kidney, liver, and lung) appeared normal (data not shown).

Prolonged Survival of H-2L^d-Bearing Skin Allografts. Skin grafting was performed to determine whether the *in vivo* tolerance evidenced by a lack of spontaneous pancreatic infiltrates in EL transgenic mice was indeed lost in doubly transgenic mice, which developed pancreatic infiltrates associated with tissue injury. Nontransgenic mice (four of five) and 2C TCR transgenic mice (three of three) rejected H-2L^d-bearing tail skin grafts in 10–16 days following transplantation. In contrast, allografts survived 30–66 days (the upper limit of the follow-up period) in EL transgenic mice (six of seven) and doubly transgenic mice (seven of eight). Control allografts from C57BL/6 mice survived for up to 66 days in four of five nontransgenic, two of three 2C TCR transgenic, six of seven EL transgenic, and seven of eight doubly transgenic mice. Thus, prolonged allograft survival was found in doubly transgenic mice in spite of the development of lymphocytic infiltrates in exocrine pancreas. This suggests that pancreatic infiltrates may form during the course of the induction of tolerance toward H-2L^d.

Elimination of CD8$^+$2C TCR$^+$ Cells and a Reduction in Alloresponsiveness of Residual T Cells. Flow cytometric analysis of T cells from peripheral lymphoid organs was performed to further assess the extent of peripheral tolerance induction in doubly transgenic mice. This analysis revealed a loss of up to 80% of CD8$^+$2C TCR$^+$ cells (Fig. 4). At 8 weeks, the proportion of CD8$^+$2C TCR$^+$ splenic T cells was significantly reduced in doubly transgenic mice compared with 2C TCR transgenic mice (17.3 ± 7.4% vs. 48.9 ± 6.5%, $n = 6$). There were fewer CD8$^+$2C TCR$^+$ splenic T cells at 16 weeks (6.8 ± 2.4% vs. 52.0 ± 6.8%, $n = 5$). Cells from pancreatic lymph nodes (typically mildly to moderately enlarged), as well as from nonpancreatic lymph nodes of doubly transgenic mice, showed a depletion of CD8$^+$2C TCR$^+$ lymphocytes comparable to that seen for splenic T cells (data not shown). Thus, depletion of autoreactive cells may have significantly contributed to the induction of peripheral tolerance in these mice.

In addition to a significant depletion of CD8$^+$2C TCR$^+$ peripheral T cells, a substantial reduction in the *in vitro* alloreactivity of residual splenic T cells (Fig. 5) and lymph node cells from 8- to- 16-week-old doubly transgenic mice was also observed. Lymphoid cells from doubly transgenic mice responded poorly to H-2L^d-bearing stimulator cells in an *in vitro* proliferation assay, compared with cells from 2C TCR singly transgenic mice. When recombinant murine interleukin 2 (20 units/ml) was added along with H-2L^d-bearing stimulator cells in the *in vitro* proliferation assay, the alloreactivity of residual splenic T cells from doubly transgenic mice to H-2L^d was restored to levels expected in the context of a loss of up to 80% of alloreactive CD8$^+$2C TCR$^+$ cells. Thus, it appears that in addition to a loss of CD8$^+$2C TCR$^+$ cells, a reduced functional capacity of residual T cells also contributed to the tolerant state in these mice.

To determine whether the reduced functional activity of residual T cells from double transgenic mice was due to immune suppression, a 50:50 mixture of splenic T cells from 2C TCR and doubly transgenic mice was made and tested in the *in vitro* proliferation assay. There was no evidence of clonal suppression, as the extent of proliferation of mixed splenic T cells did not differ from that seen for T cells from 2C TCR transgenic mice (data not shown).

DISCUSSION

A doubly transgenic mouse model system was established to study the fate of T cells whose receptor specificity is directed against a peripherally located antigen. H-2L^d served as the peripheral self-antigen and was expressed on pancreatic acinar cells, while transgenic T cells selectively expressed 2C TCR (12), an antigen receptor specific for H-2L^d.

Normal Thymocyte Differentiation. Thymocyte differentiation was unaffected by the presence of H-2L^d on exocrine pancreas. The relative proportion of thymocyte subsets was similar for doubly transgenic and 2C TCR transgenic mice. 2C TCR-bearing thymocytes were positively selected without evidence of negative selection. Thus, autoreactive thymocytes can differentiate in the context of a relatively large reservoir of peripheral self-antigen without clonal deletion in the thymus.

Pancreatic Lymphocytic Infiltrates in Doubly Transgenic Mice. A consistent finding of prior studies using peripherally expressed self-antigen in a singly transgenic mouse model has been the absence of spontaneous organ infiltration or injury when the self-antigen is expressed extrathymically prior to

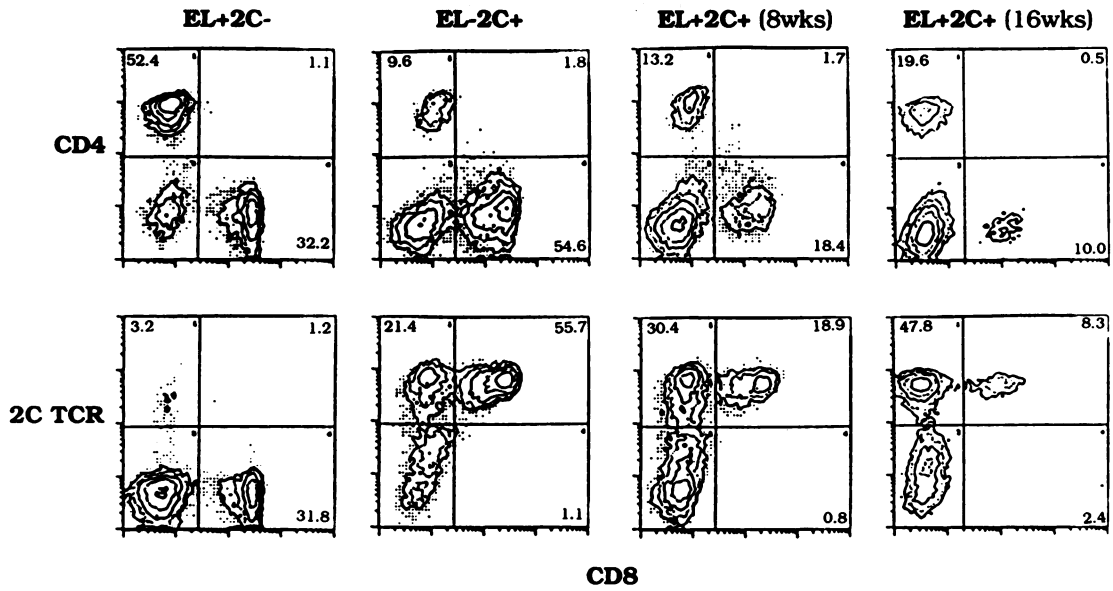

FIG. 4. Peripheral depletion of CD8+2C TCR+ splenic T cells is detectable in doubly transgenic mice. Representative flow cytometric analyses of splenic T cells from 8-week-old EL (EL+2C−), 2C TCR (EL−2C+), and doubly (EL+2C+) transgenic mice and 16-week-old doubly transgenic mice.

birth (5, 6, 9, 11, 19–24). Likewise, EL singly transgenic mice did not develop spontaneous autoimmunity in exocrine pancreas. In contrast, the exocrine pancreas of doubly transgenic mice developed a progressive lymphocytic infiltration associated with organ injury. If this infiltrate were due to the presence of an appreciable number of activated autoreactive cells within the pancreas, we would have expected lymph nodes draining the organ to have an increase in the number of autoreactive T cells as well as an increase in the responsiveness of lymph node cells to antigen presented *in vitro*. Lymph nodes draining the pancreas of doubly transgenic mice were analyzed and found to have a significant depletion of CD8+2C TCR+ cells. Since examination of lymph nodes draining the pancreas showed that not all clonotype-bearing T cells were eliminated, the remaining autospecific T cells must have been rendered anergic prior to exiting the pancreas. These anergic cells do not express increased surface levels of Pgp-1, an activation marker, suggesting that they are not rendered unresponsive subsequent to being fully activated (see below). Thus, it is likely that a significant proportion of autoreactive T cells are made tolerant during the course of their initial interaction with pancreatic tissue, but not before pancreatic injury has been induced. Further study will be required to determine whether tissue injury was induced by a few autospecific T cells that escaped tolerization and were activated *in situ* (but not detectable in lymph nodes draining the pancreas) or whether injury was a result of local release of the contents of cytotoxic granules occurring during the elimination of nonactivated CD8+2C TCR+ T cells *in situ* (an innocent-bystander scenario). An additional approach in determining the mechanism of tolerance induction in this model would be to define more precisely the temporal relationship between the onset of tolerance and the development of pancreatic lymphocytic infiltrates. In preliminary studies using 2- to 4-week-old mice, we have observed that tolerance may precede the onset of gross pancreatic infiltrates (data not shown).

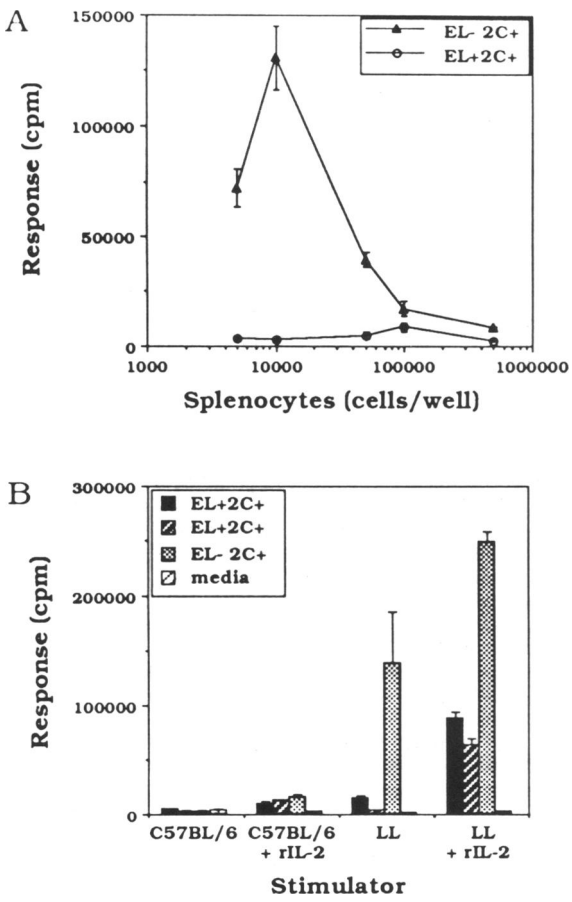

FIG. 5. (*A*) *In vitro* alloreactivity of residual splenocytes from doubly transgenic mice (EL+2C+) to irradiated Ld-bearing splenic T cells from LL transgenic mice (H-2b) was reduced compared with that of T cells from 2C TCR (EL−2C+) transgenic mice. (*B*) Recombinant murine interleukin 2 (rIL-2, 20 units/ml) restores alloreactivity of splenic T cells from doubly transgenic mice (10^5 responder cells per well).

Depletion of CD8+2C TCR+ Cells and Reduced Function of Residual T Cells. The percentage of CD8+2C TCR+ T cells was significantly reduced in the peripheral lymphoid organs of doubly transgenic mice. The elimination of up to 80% of the CD8+2C TCR+ T-cell subset could be due to deletion of these cells by a mechanism analogous to the clonal deletion of autoreactive thymocytes (25). Tolerance induction can also be associated with a down-modulation of CD8 (26, 27) or TCR

(23), neither of which has been observed here. Another mechanism of tolerance induction has been described in a system where Mls antigen is injected into adult mice that have mature Mls-reactive T cells (20–22). Elimination of Mls-reactive T cells occurred following a period of T-cell activation and clonal expansion. Evidence for activation and clonal expansion, followed by elimination of $CD8^+2C\ TCR^+$ cells, was not found in our system. Instead of clonal expansion of $CD8^+2C\ TCR^+$ cells from lymph nodes draining the pancreas, flow cytometry demonstrated clonal depletion of this population. In addition, the *in vitro* alloreactivity of residual T cells from lymph nodes draining the pancreas was significantly reduced, suggesting that a state of T-cell activation was not present. To further investigate whether evidence for T-cell activation could be found, surface levels of Pgp-1, a T-cell activation marker, were determined by flow cytometry. Pgp-1 was not found to be significantly increased on $CD8^+2C\ TCR^+$ splenocytes isolated from doubly transgenic mice compared with nontransgenic or 2C singly transgenic controls (data not shown). This finding also suggests an absence of a general state of T-cell activation.

The reduced alloreactivity of residual T cells from doubly transgenic mice is in agreement with studies that show a reduced T-cell responsiveness associated with peripheral expression of self-antigen (2–6). The reduced alloreactivity of residual T cells contributed to the *in vivo* tolerant state of doubly transgenic and EL singly transgenic mice. Functional tolerance was reversed by interleukin 2, suggesting that the mechanism of anergy induction in residual T cells may involve a down-regulation of interleukin 2 production without loss of functional surface interleukin receptor (28).

Implications of This Model System. These findings suggest that upon exiting the thymus, virgin autoreactive T cells travel to peripheral tissues, where they can be made tolerant by a process of clonal elimination and/or clonal anergy, following direct contact with self-antigen (in this case, $H\text{-}2L^d$). Interestingly, in doubly transgenic mice, an associated organ injury may be observed even though T cells become functionally tolerant. Target organ injury may have been detectable in the doubly transgenic mouse system because the number of autoreactive T cells is relatively high. Such tissue destruction may occur in singly transgenic mice but go undetected due to a lower number of autoreactive T cells that would be predicted to result in a relatively low grade of tissue injury during elimination *in situ*. Thus, infiltrates in the presenting tissue would be expected to occur in doubly transgenic mice when normal tissue repair processes fail to keep pace with the level of target organ injury.

In summary, a doubly transgenic mouse model is presented in which it is possible to track the developmental fate of specific autoreactive T cells in the context of a peripherally expressed self-antigen. Autoreactive 2C TCR-bearing lymphocytes were tolerized by a combination of clonal depletion and a reduced responsiveness of residual T cells. Although spontaneous lymphocytic infiltrates were not detected in pancreas from EL singly transgenic mice, infiltrates were noted in doubly transgenic mice. This suggests that low-grade organ injury may be occurring in nontransgenic animals, but at rates that are below the level of detection by conventional methods. That the residual T-cell population is essentially clonal should facilitate future studies of the biochemical and molecular basis of tolerance induction.

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