T Cell Tolerance to a Neo-Self Antigen Expressed by Thymic Epithelial Cells: The Soluble Form Is More Effective Than the Membrane-Bound Form

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We have previously shown that transgenic (Tg) mice expressing either soluble or membrane-bound hen egg lysozyme (sHEL or mHEL, respectively) under control of the αA-crystallin promoter develop tolerance due to thymic expression of minuscule amounts of HEL. To further address the mechanisms by which this tolerance develops, we mated these two lines of Tg mice with the 3A9 line of HEL-specific TCR Tg mice, to produce double-Tg mice. Both lines of double-Tg mice showed deletion of HEL-specific T cells, demonstrated by reduction in numbers of these cells in the thymus and periphery, as well as by reduced proliferative response to HEL in vitro. In addition, the actual deletional process in thymi of the double-Tg mice was visualized in situ by the TUNEL assay and measured by binding of Annexin V. Notably, the apoptosis localized mainly in the thymic medulla, in line with the finding that the populations showing deletion and increased Annexin V binding consisted mainly of single- and double-positive thymocytes. Interestingly, the thymic deletional effect of sHEL was superior to that of mHEL in contrast to the opposite differential tolerogenic effects of these HEL forms on B cells specific to this Ag. Analysis of bone marrow chimeras indicates that both forms of HEL are produced by irradiation-resistant thymic stromal cells and the data suggest that sHEL is more effective in deleting 3A9 T cells due mainly to its higher accessibility to cross-presentation by dendritic APC. ***The Journal of Immunology,*** **2003, 170: 3954–3962.**

The T cell repertoire is shaped mainly by the complex processes of positive and negative selection in the thymus. Although both processes have been studied extensively, many details are still not completely clear.

The negative selection process is responsible for the elimination of self-reactive lymphocytes. Because the number of T cells specific to any self-Ag is normally too low to allow examination of the deletion process, the investigation of negative selection has been conducted on lymphocyte populations responsive to superantigens (1, 2) or on homogeneous populations of T cells with transgenic (Tg)[3] TCR (2–4). Maturing thymocytes that express TCR with high affinity to self-Ags undergo apoptosis upon exposure to their specific target Ag (2–6), a process that takes place mainly in the thymic medulla and at the corticomedullary junction (4, 5, 7). Recent studies, in particular by Kyewski's group, have identified three populations of thymic cells that present self-Ags in this process. These populations include medullary and, to a lesser extent, cortical epithelial cells and hematogenous dendritic cells (8, 9). Of particular interest are the medullary epithelial cells that were found to express numerous peripheral tissue-specific Ags, such as insulin or myelin proteolipid protein, and therefore play a crucial role in preventing autoimmune processes against these tissues (8, 9).

To investigate immunotolerance toward ocular-specific Ags, we have generated Tg mice that express a foreign Ag, hen egg lysozyme (HEL), under control of the lens αA-crystallin promoter (10). These mice develop tolerance against HEL that is attributed to thymic expression of minuscule amounts of this Ag (10). Mating these HEL-Tg mice with Tg mice that express HEL-specific TCR on their T cells (designated "3A9" (11)) created double-Tg mice that allow further investigation of the process by which thymic deletion of HEL-specific T cells occurs. HEL is expressed transgenically in two forms, soluble (sHEL) and membrane-bound (mHEL) (12, 13) and studies by Goodnow et al. (12) have shown that the two forms differ remarkably in their tolerogenic effects on HEL-specific B cells. Whereas Tg expression of sHEL induced merely anergy in the HEL-specific B cell population (12), expression of mHEL produced tolerance by actually deleting these B cells (13). A more recent study by Goodnow's group (14) and data collected in the present study show, however, that unlike with the B cell population, both forms of HEL produced deletion of T cells. Previous studies assessing T cell recognition of mHEL and sHEL have used Tg mice in which the two forms of HEL were expressed under different promoters (14). In our current study, however, we expressed both forms of HEL under control of the same promoter and, therefore, we could compare tolerance induction between the two forms of the Ag. We show here that sHEL is superior to mHEL in its capacity to delete HEL-specific T cells. The superiority of the soluble form of HEL in inducing immunotolerance was established by several parameters. Moreover, findings recorded here support the notion that Ag presentation by thymic stromal

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[3] Abbreviations used in this paper: Tg, transgenic; HEL, hen egg lysozyme; mHEL, membrane-bound HEL; sHEL. soluble HEL; WT, wild type; SP, single positive; DP, double positive.

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cells is not highly effective for tolerance induction and that cross-presentation is a major mechanism in thymic deletion.

Materials and Methods

Mice

mHEL-Tg or sHEL-Tg mice in which the transgene is expressed under the αA-crystallin promoter were generated as detailed elsewhere (10) and maintained in our facility on the FVB/N background. HEL-specific TCR Tg mice, on the B10.BR background, designated 3A9 (11), were a generous gift from Dr. M. Davis (Stanford University, Stanford, CA). Mice from each of the two HEL-Tg lines were mated with 3A9 mice to produce (FVB/N × B10.BR)F_1 hybrids, expressing no transgene (wild type (WT), either one of the three transgenes (3A9, mHEL, or sHEL), or two transgenes ("mHEL double Tg" or "sHEL double Tg"). Only such F_1 hybrid mice were used in all experiments of the present study.

Bone marrow chimeric mice were generated by irradiating recipient mice, as indicated, with 9.5 Gy, followed by i.v. injection with 5×10^6 donor bone marrow cells as indicated. Cells from the chimeric mice were examined 6 wk later.

The mice were housed in a pathogen-free facility and all manipulations were conducted in compliance with the National Institutes of Health Resolution on the Use of Animals in Research.

HEL measurement

Spleens from Ig-HEL-Tg mice, expressing IgM and IgD Abs/receptors for HEL (12), were minced through a cell strainer. The RBC were lysed using an ammonium chloride based solution. Then 0.5×10^6 cells were incubated for 30 min at 4°C with 50 μl of diluted blood serum or PBS extracts of eyes from 6-wk-old mice of the two Tg lines. The cells were washed three times and stained with anti-B220-FITC and HY-9-Tricolor conjugate (15), which specifically binds HEL at a different nonoverlapping epitope from the Ig-HEL transgene. Quantification was performed relative to a standard curve generated by measuring the level of soluble HEL binding. Specificity of HEL binding was assessed on splenocytes from non-Tg mice. Samples were acquired on a FACScan cytometer (BD Biosciences, Mountain View, CA) using CellQuest and analyzed on FlowJo software.

Quantitative RT-PCR analysis

First-strand synthesis was performed as described previously (16) using thymi of mHEL-Tg or sHEL-Tg mice, either untreated or 4 days following whole-body irradiation with 6.0 Gy. A negative control reaction without reverse transcriptase was performed for each RNA sample. RNA samples were normalized to 18S RNA using a TaqMan Ribosomal RNA Control Reagents kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI 7700 (Applied Biosystems) or ICycler iQ Real Time (Bio-Rad, Hercules, CA) Sequence Detection System with HEL-specific primers and probe: HEL forward primer, 5′-CGTGCTCAGCCCTGCTG-3′; HEL reverse primer, 5′-GCCGTTTCCATCGCTGAC-3′; and HEL hybridization probe, 6FAM-CGAGCGTGAACTGCGCGAAGAAGTAMRA. PCR primers and probes for GAPDH are described in the TaqMan Rodent GAPDH Control Reagents kit (Applied Biosystems). PCR parameters are as recommended for the TaqMan Universal PCR master mix kit (Applied Biosystems). Triplicate samples of 10-fold serial dilutions of cesium chloride-banded HEL plasmid cDNA were assayed and used to construct the standard curves.

Flow cytometry analysis

Directly labeled Abs specific for CD4, CD8, and Thy1.2 were purchased from BD PharMingen (San Diego, CA). The clonotype-specific Ab 1G12 that recognizes 3A9 T cells was a generous gift from Dr. E. Unanue (Washington University, St. Louis, MO). Single-cell suspensions were prepared and analyzed as previously described (17). Analysis of splenocytes and thymocytes from Tg mice was performed on a FACScan cytometer (BD Biosciences). Ten thousand events in a live gate were acquired although all ungated events were saved for later analysis.

Lymphocyte proliferation assay

Spleen or lymph node cells were collected and tested for their proliferative response against different HEL concentrations as described elsewhere (18), with the exception that the cultures were incubated for a total of 72 h. IL-2 (Chiron, Emeryville, CA) was added to certain cultures, as indicated, at 100 U/ml. Certain cultures were made of mixtures of naive splenocytes of WT mice and 3A9 mice as indicated.

Cellular proliferation assay via dye dilution

Spleen cells were washed extensively in PBS to remove residual protein, then 10^7 cells/ml were incubated in 1 μM CFSE (Molecular Probes, Eugene, OR) for 8 min at room temperature as previously described (19). Labeling reaction was quenched with an equal volume of FBS and the cells were centrifuged and washed three times in complete tissue culture medium consisting of 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, 100 μM nonessential amino acids, 1 mM sodium pyruvate, and 25 μM 2-ME in RPMI 1640 (BioWhittaker, Walkersville, MD). CFSE-labeled cells were diluted in complete tissue culture medium and were cultured at 4×10^5 in 96-well U-bottom plates in the absence or presence of titrated HEL. Cultures were harvested after 72 h of incubation at 37°C in 5% CO_2, stained with biotinylated 1G12 Ab and PE-labeled CD4 Ab for 30 min at 4°C, washed three times in FACS buffer (0.2% BSA, 0.01% sodium azide in HBSS without phenol red), and then incubated with avidin-CyChrome (BD PharMingen) for 10 min, followed by extensive washing in FACS buffer. $CD4^+$ $1G12^{high}$ and $CD4^+Ig12^{int}$ cells were then gated and analyzed for dye dilution peaks. Cells were analyzed on a FACScan cytometer (BD Biosciences). Ten thousand events in a live gate were acquired although all ungated events were saved for later analysis.

Detection of apoptotic thymocytes ex vivo

Single-cell suspensions were resuspended in FACS buffer, then 1×10^6 cells were stained with the desired directly conjugated Ab for 30 min at 4°C, washed three times in FACS buffer, and then incubated with avidin-CyChrome (BD PharMingen) for 10 min, followed by extensive washing in FACS buffer. Cells were analyzed on a FACScan cytometer (BD Biosciences). Ten thousand events in a live gate were acquired although all ungated events were saved for later analysis. To detect apoptotic thymocytes, cells were then resuspended in annexin-binding buffer (BD PharMingen) immediately before analysis and labeled with Annexin V-PE per the manufacturer's instructions (BD PharMingen) with the exception that Annexin V was diluted 1/5 rather than used undiluted.

TUNEL assay

The level of in situ apoptosis in mouse thymi was evaluated by the Trevigen Apoptotic Cell System (TACS) using the TACS 2 TdT Blue Label in situ Apoptosis Detection kit (Trevigen, Gaithersburg, MD). Briefly, Formalin-fixed, paraffin-embedded sections of mouse thymi were deparaffinized in xylene, then hydrated in graded ethanol concentrations. The protein was digested using cytopore (Trevigen) and then endogenase peroxide was blocked with 2% H_2O_2. The tissue was equilibrated with labeling buffer, then incubated with the labeling reaction mix for 45 min. The labeling reaction was terminated with stop buffer (Trevigen), then the sections were incubated with streptavidin-HRP conjugate. The sections were stained with Blue Label, followed by Red Counterstain C. The sections were dehydrated in graded ethanol, followed by xylene, then mounted with Permount (Fisher Scientific, Fairlawn, NJ). The number of apoptotic cells were counted in five different fields at ×40 magnification, in a masked fashion, then averaged. Two to five mice were examined for each tested group.

Results

Phenotypes of the HEL-Tg mouse lines: eye morphology and HEL expression in the eyes and thymus

Fig. 1 shows sections of eyes of sHEL- and mHEL-Tg mice. Whereas the sHEL-Tg mouse eyes exhibit normal morphology (Fig. 1*A*), eyes of the mHEL-Tg mice are dystrophic and much smaller in size, with disruption of the lens fibers and distortion of the lens capsule (Fig. 1*C*). No inflammation was detected, however, in eyes of either one of these single Tg mice.

Since both mHEL and sHEL genes were expressed in the present study under control of the αA-crystallin promoter, the main depot of HEL was found in the lens of these mice. Measurements of HEL in eyes and serum of the two lines of HEL Tg are summarized in Table I. The total HEL amount per eye was moderately higher in the mHEL-Tg mice than in the sHEL-Tg mice (232 vs 162 ng/eye), but the HEL concentration per milligram total protein was remarkably higher in the mHEL-Tg mice (545 vs 63 ng HEL/mg protein), since eyes of mHEL-Tg mice are dystrophic and contain much smaller amounts of total protein than eyes of

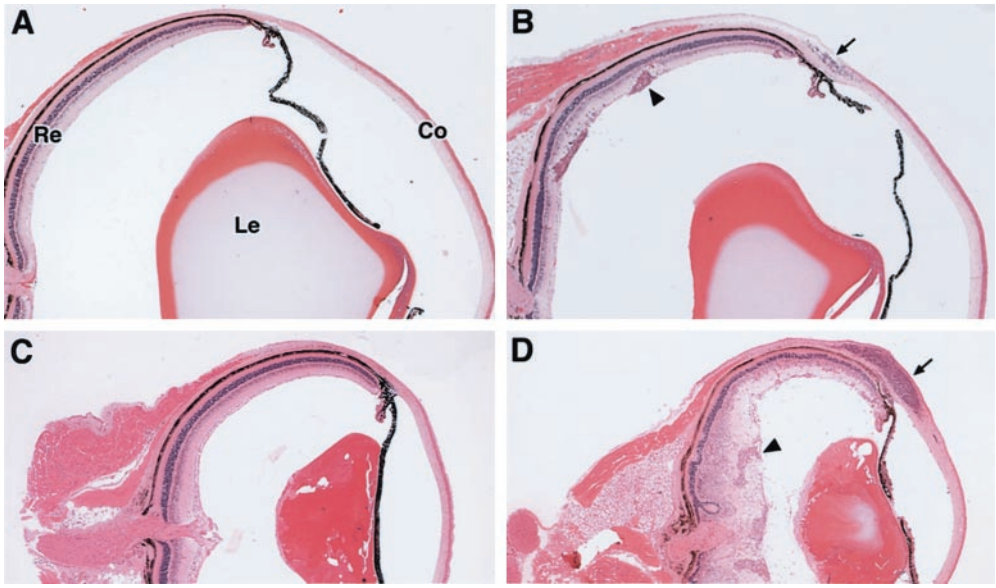

FIGURE 1. Histological sections of eyes from single HEL-Tg and double-Tg mice at 6–8 wk of age. *A*, sHEL-Tg mouse eye showing the typical morphology of normal eye tissues, Co, cornea; Le, lens; Re, retina. *B*, sHEL double-Tg mouse eye showing mild local lymphoid cell accumulation in the limbus (arrow) and around retinal blood vessels (arrowhead). *C*, mHEL-Tg mouse eye demonstrating the characteristic abnormal morphology of this mouse line (see Ref. 10): the eye is dystrophic and remarkably smaller than normal, the typical morphology of the lens is completely disrupted, and its capsule is distorted. *D*, mHEL double-Tg mouse eye showing intense lymphoid infiltration of the limbus (arrow) and retinal blood vessels (arrowhead) as well as severe retinal edema. H&E; magnification, ×50.

sHEL-Tg mice (0.42 and 2.67 mg/eye, respectively). The two HEL-Tg lines also differed in their serum HEL content. Interestingly, in contrast to their lower HEL expression in the lens and intact lens capsule, sHEL-Tg mice had measurable amount of HEL in their serum, whereas no serum HEL was detected in the mHEL mice (Table I).

HEL-Tg mice expressing HEL under control of the αA-crystallin promoter express the transgene in the thymus as well (10). Since the HEL protein levels are below the assay's threshold, the presence of HEL in the thymus of the two HEL-Tg mouse lines was assessed according to the level of the corresponding HEL mRNA molecules using the real-time quantitative PCR assay. As seen in Fig. 2, the level of HEL mRNA transcripts in the mHEL-Tg mice was higher than that in the thymi of the sHEL-Tg mice by >50%. It should be noted that standard curves generated from the HEL cDNA dilution series showed excellent linearity, indicating a precise, quantitative relationship between cDNA copy number and fluorescence signal intensity within the dynamic range of the assay (data not shown).

Table I. *HEL in eyes and serum of sHEL- and mHEL-Tg mice*[a]

Mice	Eyes			Serum HEL (ng/ml)
	Total protein (mg/eye)	HEL content (ng/eye)	ng HEL/mg protein	
sHEL Tg	2.55	162	63	3.0[b]
mHEL Tg	0.42	232	545	Undetectable[c]

[a] Whole eye extracts or serum samples of 6-wk-old mice of the two lines were examined. Protein levels were measured by the Coomassie Plus Protein Assay Reagent (Pierce), while HEL concentrations were determined as detailed in *Materials and Methods*. The recorded data are from pools of two eye extracts or means of three individual mouse serum samples.

[b] All three mouse serum samples gave the same value.

[c] The method's sensitivity threshold is 1.0 ng/ml.

Phenotypes of the double-Tg mice: development of ocular inflammation

Double-Tg mice, created by mating the TCR-Tg 3A9 mice with either mHEL or sHEL single Tg mice, developed inflammatory ocular changes. These changes included in sHEL double-Tg mice moderate accumulation of inflammatory cells in the limbus, as well as perivascular cellular infiltration in the retina (Fig. 1*B*), seen in some mice of this group. Remarkably, more severe changes were seen in the mHEL double-Tg mice, with intense cellular infiltration in the limbus, retina. and vitreous as well as severe retinal edema (Fig. 1*D*). The histological changes in both groups of double-Tg mice peaked between 1 and 3 mo of age and gradually decreased thereafter.

Double-Tg mice resembled their corresponding parental HEL-Tg mice in their HEL levels (data not shown). As detailed below, the expression of HEL in the double-Tg mice affected profoundly their T cell populations.

Deletion of HEL-specific T cells in sHEL and mHEL double-Tg mice

To characterize the fate of HEL-specific T cells in the HEL double-Tg mice, we assessed their spleen content of 3A9 cells using the 1G12 clonotypic Ab. Spleen cells were gated for Thy1.2 expression, then assessed for expression of 1G12 and CD4. The number of 1G12-positive cells recovered in these mice was determined from total splenic cell recovery by the percentage of 1G12-positive cells. Both sHEL and mHEL double-Tg mice exhibited significant losses of $CD4^+$ "clonotype high" ($1G12^{high}$) cells (quadrant R3) compared with 3A9 controls (Fig. 3*A*). Interestingly, sHEL double-Tg mice also exhibited loss of $CD4^+$ "clonotype intermediate" ($1G12^{int}$) cells (quadrant R2) as well, whereas numbers of these cells were comparable between mHEL double-Tg and 3A9 mice. These findings demonstrate that elimination of clonotype-expressing cells is more complete in sHEL double-Tg mice than in mHEL

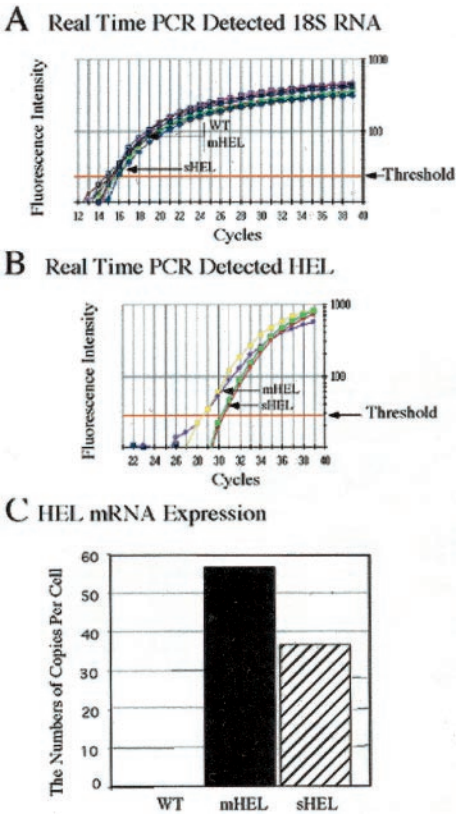

FIGURE 2. Real-time PCR measurement of HEL mRNA in thymi of mHEL- and sHEL-Tg mice. RNA samples of two pooled thymi of each of the Tg mice and WT controls were used as detailed in *Materials and Methods*. *A*, Results with the 18S probe/primers. *B*, Data with the HEL probe/primers. All tests were duplicated, with each measurement recorded here by a different color. The copy numbers per cell, recorded in *C*, were calculated against a standard curve made from the known copy numbers of the HEL-cDNA plasmid.

double-Tg mice, with both high and low $1G12^+$ cells decreased in sHEL double-Tg mice, whereas deletion in the mHEL double-Tg mice is less complete, allowing cells with low expression of 3A9 TCR to escape deletion.

Although the main depot of HEL in the double-Tg mice is in the eye, minute amounts of HEL are expressed in thymi of these mice and our previous study suggested that deletion of HEL-specific T cells occurs mainly in the thymus (10). We examined, therefore, the thymic populations in the double-Tg mice and compared them to those in 3A9 mice. Both lines of double-Tg mice exhibited remarkably lower number of total thymocytes than in the 3A9 mice (mean numbers per thymus, with three mice per group ($\times 10^6$): 10.1, 19.9, and 65.8 in sHEL double Tg, mHEL double Tg, and 3A9, respectively). FACS analysis of thymocyte populations, shown in Fig. 3*B*, revealed that the loss of cells in the double-Tg mice was greatest in the single-positive (SP, "CD4") subpopulation and the double-positive (DP) subpopulations.

Decreased lymphocyte responses to HEL in double-Tg mice

Naive lymphocytes of 3A9 mice proliferate vigorously upon stimulation with HEL in culture. Lymphocytes from the double-Tg mice also responded to HEL, but with lower intensity. As shown in Fig. 4, the reduced response was exhibited by lymphocytes from both lines of double-Tg mice, but the reduction was particularly clear in the sHEL double-Tg mice. Also of note is the observation that the reduced responsiveness became in general more apparent with age (Fig. 4). It is particularly interesting that the reduced response of the double-Tg mice was mostly pronounced at the lower range of HEL concentrations; the lowest HEL concentrations to stimulate mHEL or sHEL double-Tg lymphocytes were higher than those of 3A9 cells by at least 1 or 2 orders of magnitude, respectively.

To learn whether the lack of response of double-Tg mice at the low HEL concentrations is due to a lower number of responding

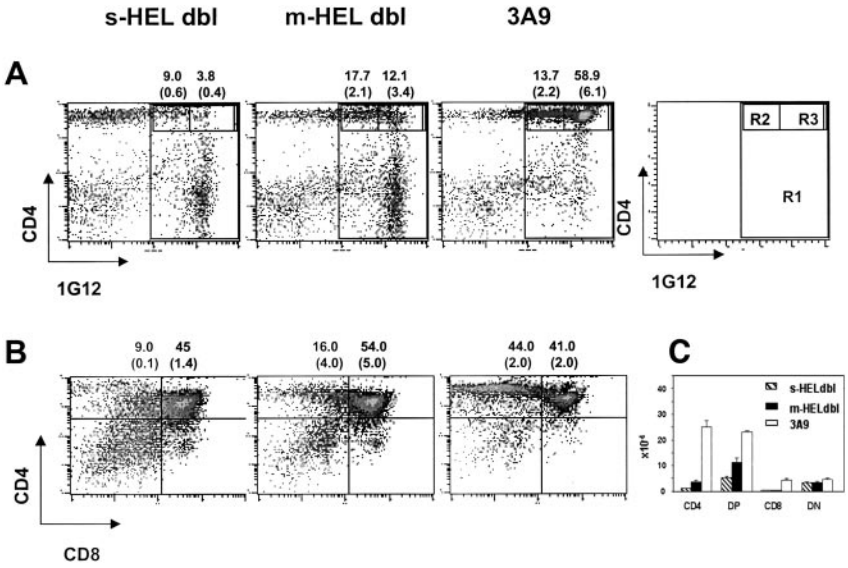

FIGURE 3. Selective loss of HEL-specific lymphocytes in the spleen and thymus of double (dbl)-Tg mice. FACS analysis of splenocytes (*A*) and thymocytes (*B*) from double-Tg mice and 3A9 controls. Profiles are representative of the analysis of three individual mice from each group. *A*, Analysis of CD4 and 1G12 expression of Thy1.2-expressing cells reveals a selective loss of the $CD4^+1G12^{high}$ T cells (*quadrant R3*) by the double-Tg mice. sHEL double-Tg mice, but not mHEL double-Tg mice, also show a decrease in the $CD4^+1G12^{int}$ subpopulation (*R2*). No significant change in cell numbers was observed, however, in the $CD4^-1G12^+$ (*R1*) T cells of the double-Tg mice. The recorded values show mean actual number of each subpopulation ± SEM. *B*, Analysis of CD4 and CD8 expression by thymocytes reveals profound losses of SP (CD4) thymocytes in the double-Tg mice as compared with 3A9 controls, with sHEL double-Tg mice being more affected than the mHEL double-Tg mice. The recorded values are calculated percentages of each subpopulation ± SEM. The actual cell numbers in each subpopulation of the three mouse lines are recorded in *C*.

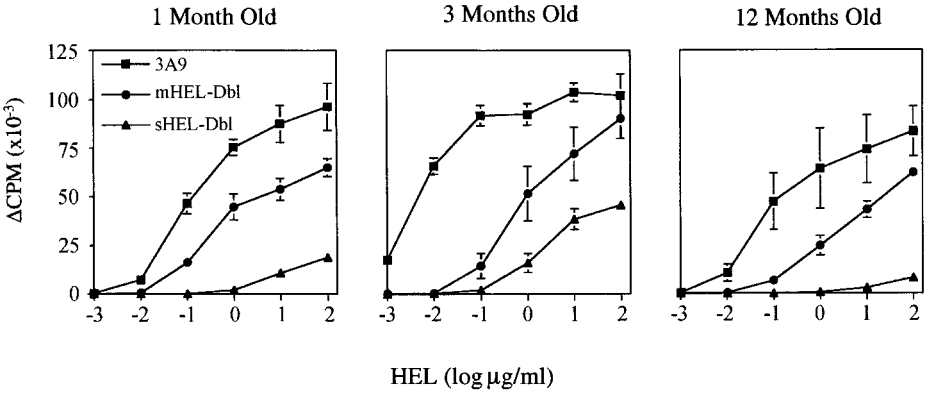

FIGURE 4. Reduced lymphocyte proliferation to HEL in double (Dbl)-Tg mice. Cultured spleen cells from the two lines of double-Tg mice, at the indicated ages, were compared with their single-Tg 3A9 control cells for response to different HEL concentrations. Cultures consisted of 3×10^5 spleen cells, in 0.2 ml, incubated for a total of 3 days and pulsed with [^{3}H]thymidine for the last 16 h. The presented data are mean Δcpm ± SEM of responses of three mice of each mouse line, at each tested age, in three different experiments.

cells or to a selective elimination of lymphocytes with high affinity, we compared the dose response of sHEL double-Tg mouse spleen cells to that of mixed populations of naive splenocytes from 3A9 and WT mice, at different ratios of the two cell types. As seen in Fig. 5*A*, the mixed cell populations responded to HEL at the low concentrations of 0.01 and 0.1 μg/ml that had minimal or no stimulatory effect on lymphocytes of the double-Tg cells. On the other hand, the double-Tg cells responded stronger than most cell mixtures when cultured with the high HEL concentration of 10 μg/ml.

To further address whether the decreased response of double-Tg mice was attributable to a selective loss of lymphocyte population capable of responding to low HEL concentrations, we used dye dilution analysis to directly visualize the proliferative response of $1G12^+$ cells in the double-Tg mice. CFSE-labeled spleen cells from the two double-Tg lines and 3A9 mice were cultured with serial concentrations of HEL and the dye dilution analysis was conducted on gated subpopulations, $1G12^{high}$ (R1) and $1G12^{int}$ (R2), of the three mouse lines. Data collected with cultures stimulated with HEL at 100 or 10 ng/ml are recorded in Fig. 5*B*. Although both subpopulations of 1G12-positive cells from mHEL double-Tg mice responded well and similarly to 3A9 cells when cultured with HEL at 100 ng/ml, these mHEL double-Tg subpopulations responded less vigorously than the two subpopulations of 3A9 at 10 ng/ml. Interestingly, the $1G12^{high}$ subpopulation (R1) of mHEL double-Tg mice multiplied more than the $1G12^{int}$ (R2) cells. mHEL double-Tg cells failed to respond to HEL at 1 ng/ml, whereas 3A9 cells did, with the $1G12^{high}$ cells of these mice responding better than the $1G12^{int}$ cells (data not shown). Only a small number of 1G12-positive cells was retrieved from spleens of sHEL double-Tg mice and these cells did not respond at all to HEL at these low concentrations (Fig. 5*B*).

These two observations recorded in Fig. 5 thus support the notion that the lowered response of the double-Tg mice is because of the deletion of high-affinity clones from the repertoire.

The potential role of anergy in the reduced response to HEL of double-Tg mice was examined by adding exogenous IL-2 to HEL-stimulated cultures; anergic cells do respond in the presence of exogenous IL-2 (20). As seen in Fig. 6, a moderate enhancement of the response to HEL was repeatedly seen in IL-2-supplemented cultures of the sHEL double-Tg mice, but not in those of the mHEL double-Tg or the 3A9 controls. This finding suggests that the reduced response to HEL in sHEL double-Tg mice is partially attributable to development of anergy to HEL in these mice, but not in mHEL double-Tg mice.

Elevated apoptosis in the thymus of double-Tg mice

To test whether the loss of cells in thymi of double-Tg mice is because of deletion, we examined the thymic population of these mice for the binding of Annexin V, a marker for dying cells (21). Levels of Annexin V^+ cells were elevated in both lines of double-Tg mice over those observed in 3A9 mice (Fig. 7A, "total" cells). Numbers of Annexin V^+ cells in 3A9 thymi resembled those in WT mice or in single HEL-Tg mice (data not shown).

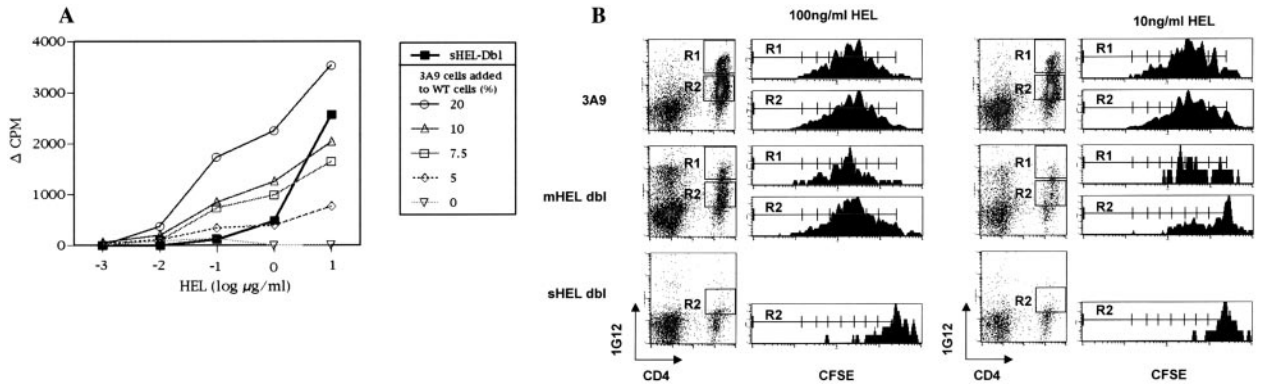

FIGURE 5. Selective loss of responsiveness to low concentrations of HEL by lymphocytes from the double-Tg mice. *A*, Proliferative responses to HEL at different concentrations by sHEL double-Tg spleen cells and by mixed cultures of splenocytes from WT and differing proportions of 3A9 mice as indicated. The data are mean Δcpm of triplicate cultures of one experiment; two repeated experiments yielded similar responses. *B*, Dye dilution analysis of the proliferative response to HEL of lymphocyte subpopulations of the three mouse lines. Pooled spleen cells were CFSE-labeled and cultured for 3 days with HEL at the indicated concentrations. Following incubation, the cells were stained with 1G12 and CD4 Abs and were FACS analyzed as shown. Dye dilution analysis was conducted on the gated subpopulations R1 and R2 as indicated. mHEL double-Tg cells resembled 3A9 lymphocytes in their response to HEL at 100 ng/ml, but were inferior in their response to HEL at 10 ng/ml. sHEL double-Tg cells did not respond to HEL at these low concentrations.

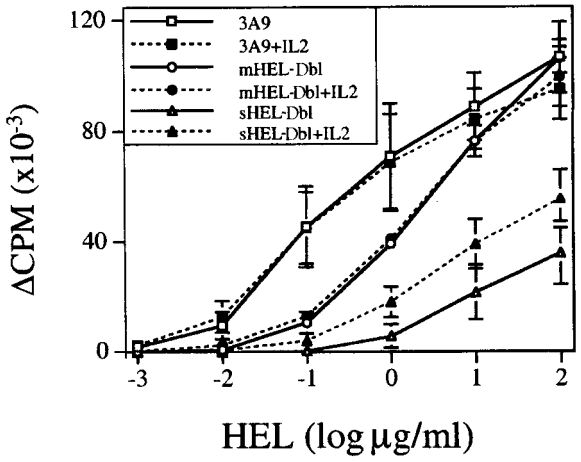

FIGURE 6. A moderate level of anergy to HEL in sHEL double (Dbl)-Tg mice. Spleen cultures of the two lines of double-Tg mice and their 3A9 controls were cultured with or without IL-2, at 100 U/ml, along with HEL at different concentrations and tested for proliferation as described in the legend for Fig. 4. Exogenous IL-2 elevated the response only in cultures of the sHEL double-Tg mice. This figure summarizes a typical experiment; the same observations were made in four other repeated experiments.

Further analysis of apoptosis in thymocyte subsets revealed a dramatic increase in apoptosis in CD4 SP thymocytes from the sHEL double-Tg mice and a lower but substantial increase in apoptosis of these cells among the mHEL double-Tg mice. The percentage of Annexin V^+ thymocytes also increased in DP thymocytes, with higher levels present in sHEL double-Tg mice as compared with mHEL double-Tg mice. Notably, no difference in apoptosis levels were observed in the double-negative population compared with 3A9 controls. These results demonstrate that, indeed, clonal deletion is ongoing in thymi of the double-Tg mice as early as the DP stage in differentiation and is more prevalent in the transgenics with sHEL than in mHEL.

Analysis of thymic deletion by the TUNEL method

The TUNEL method that identifies apoptotic cells (22) provided us with an additional approach to evaluate the level of thymic apoptosis and, importantly, enabled us to localize the thymic areas in which the enhanced apoptosis takes place. Fig. 7*C* demonstrates typical intense TUNEL reaction in the thymus of sHEL double-Tg mice. The elevated apoptosis in thymi of double-Tg mice was quantified by counting TUNEL-positive cells in thymus sections of the three mouse lines collected at three different ages. The data, summarized in Fig. 7*B*, show that the apoptosis level in the 3A9 control mice was profoundly lower than in the double-Tg mice and that cell death in sHEL double-Tg thymi was higher than that in the mHEL double-Tg mice. Of particular interest is the observation that apoptotic cells in thymi of the double-Tg mice localized mainly in the medulla (Fig. 7, *C* and *D*), unlike the even distribution of the lower numbers of apoptotic cells throughout the thymus in 3A9 mice (data not shown). The selective localization of apoptotic cells in the medulla of double-Tg mice is further underscored by our finding that the enhanced apoptosis in 3A9 mice injected systemically with HEL localized mainly in the thymic cortex (Fig. 7*E*).

HEL is produced by thymic stromal cells

One possible explanation for the surprising ability of the sHEL transgenics to develop more extensive tolerance than mHEL transgenics could be that the APC that presents HEL is different when the HEL is soluble vs membrane bound. Thymic epithelial cells were shown to be the producers of Ags specific to peripheral organs (8, 9) and it has been proposed that Ags they release are

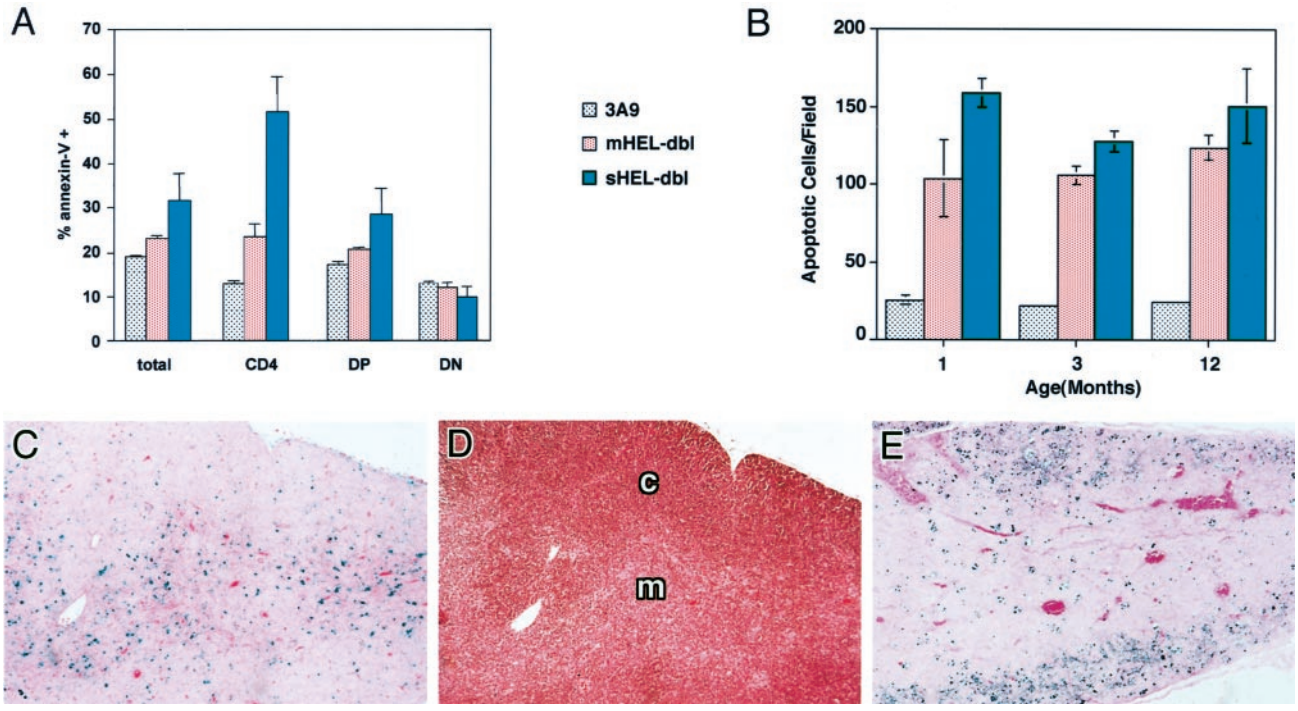

FIGURE 7. Increased apoptosis in double (dbl)-Tg mouse thymi. *A*, Annexin V binding by thymocyte subsets from the double-Tg mice and 3A9 controls. The bars represent means ± SEM of three mice per group. *B*, Counts of apoptotic cells in thymic sections of the different mouse lines at different ages. The bars represent mean counts of TUNEL-positive cells ± SEM of two to five mice in each group, with five fields monitored in each thymus (1 field = 0.25 mm^2), *C*, TUNEL reaction on a thymus section of a sHEL double-Tg mouse showing localization of intense apoptosis in the medulla. *D*, H&E staining of a serial section of the thymus shown in *C*. *E*, TUNEL reaction of the thymus from a 3A9 mouse 24 h following i.v. injection of 0.1 mg HEL.

captured by bone marrow-derived dendritic cells, the highly potent inducers of tolerance via clonal deletion (23, 24). To determine the cellular source of HEL in thymi of HEL-Tg mice, bone marrow chimeras were created in which bone marrow from the two lines of double-Tg mice and from 3A9 controls were transferred into groups of lethally irradiated WT recipients, while bone marrow from 3A9 donors was injected into irradiated sHEL-Tg or mHEL-Tg mice.

Analysis of splenic T cells, 6 wk after reconstitution (Fig. 8*A*), revealed a decrease in the recovery of $CD4^+$ clonotypic^{high} cells (quadrant R3) when HEL transgenics served as the recipients (Fig. 8*A*). Moreover, deletion of these cells was notably more effective in 3A9→sHEL Tg (1.1×10^6 cells) than in 3A9→mHEL Tg (20.8×10^6 cells). On the other hand, the recovery profiles of clonotypic^{high} cells when either one of the double-Tg mice was used as bone marrow donors (54.3×10^6 and 34.2×10^6 cells, respectively) resembled those in recipients of 3A9 donors (46.5×10^6). This pattern of differences among the various bone marrow chimeras was also observed when 1G12 levels were measured on spleen cells of these mice (Fig. 8*B*). No significant differences in cell numbers were observed in either the $CD4^+1G12^{low}$ (quadrant R2) or $CD4^-1G12^+$ (quadrant R1) subpopulations of the different chimeras (Fig. 8*A*).

The selective deletion of HEL-specific lymphocytes in chimeras with HEL-expressing thymi was also demonstrated by the pattern

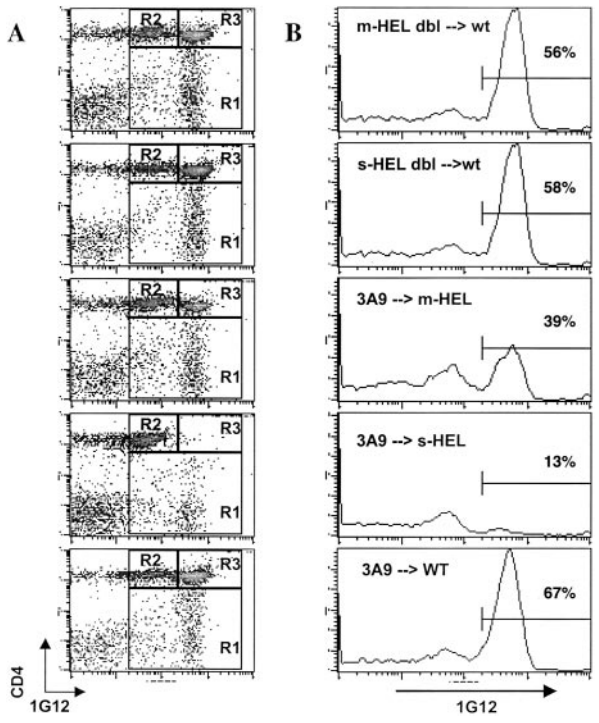

FIGURE 8. Loss of $1G12^+$ cells in bone marrow chimeras in which the recipients are HEL-Tg. Chimeras were generated and spleen cells of individual mice were analyzed for 1G12 and CD4 expression on Thy1.2 cells as described in the text. This figure shows profiles of mice representative of three mice in each group. *A*, FACS analysis showing loss of $CD4^+1G12^{high}$ cells (*quadrant R3*) only in HEL-Tg recipients of 3A9 bone marrow. The loss is greater in sHEL-Tg hosts than in mHEL-Tg hosts. No significant change in cell numbers is observed in these recipient mice in either the $CD4^-1G12^+$ (*quadrant R1*) or $CD4^+1G12^{int}$ (*quadrant R2*). The actual numbers of cells in each subpopulation are recorded in the text. *B*, Levels of 1G12 on splenocytes of the individual representative mice depicting the greater loss of 1G12 cells in sHEL-Tg hosts than in mHEL-Tg hosts.

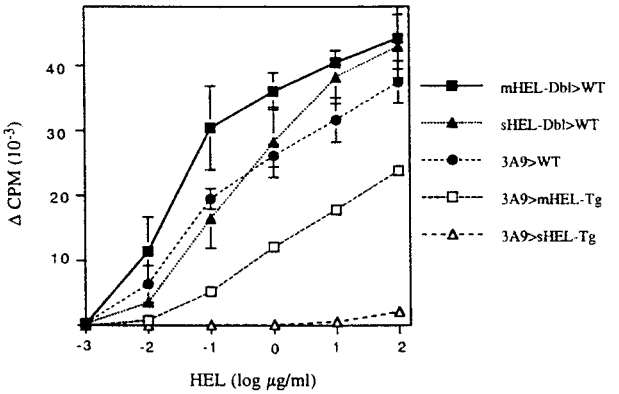

FIGURE 9. Proliferative responses to HEL of splenocytes from the bone marrow chimeras. Each group of chimeras consisted of three mice whose spleen cells were tested individually for their response to the indicated HEL concentrations. The recorded data represent mean Δcpm ± SEM of each mouse group. Reduced responses are seen only in HEL-Tg recipients of 3A9 bone marrow and are particularly apparent in the sHEL-Tg hosts.

of lymphocyte proliferation (Fig. 9). Vigorous responses to HEL, with similar dose-response curves, were demonstrated by all three groups of chimeras in which WT mice served as the recipients for bone marrow from 3A9 or the two lines of double-Tg mice. On the other hand, mHEL-Tg recipients of 3A9 bone marrow responded remarkably lower than the WT recipients and almost no response was observed in sHEL-Tg recipients.

Taken together, these results indicate that the HEL responsible for thymic deletion of 3A9 cells is not produced by bone marrow cells, but rather is expressed by radiation-resistant stromal cells in thymi of the HEL-Tg mice.

The production of HEL by radiation-resistant stromal cells was further demonstrated by measuring HEL mRNA in thymi of mHEL and sHEL mice 4 days following irradiation with 6.0 Gy using real-time PCR. The numbers of copies per cell were 234 ± 44 and 125 ± 22 in mHEL and sHEL mouse thymi, respectively; namely, approximately four times higher than those measured in thymi of the corresponding intact mice, as recorded in Fig. 2. It is also noteworthy that in line with the observation with thymi of intact mice, the mRNA values are significantly higher in mHEL-Tg than in sHEL-Tg mice.

Discussion

We have shown here that when being expressed transgenically in mouse thymi, the soluble form of HEL deletes HEL-specific T cells remarkably more efficiently than the membrane-bound form of this Ag. The difference in deletion efficiency was assessed in double-Tg mice expressing either one of the two forms of HEL in the thymus and HEL-specific TCR on most T cells. The levels of deletion were measured by several parameters: 1) counting apoptotic cells by the TUNEL technique in thymic sections or flow cytometric analysis of Annexin V binding; 2) measuring reduction in the number of cells expressing the clonotypic TCR in the thymus and spleen; and 3) the decrease in lymphocyte responsiveness to HEL. The superior deletional capacity of sHEL over that of mHEL is consistent in all of these parameters. Moreover, the superiority of sHEL in this system is also indicated by the finding that its level of thymic mRNA was lower than that of mHEL (Fig. 2), suggesting that the level of HEL produced in thymi of sHEL-Tg mice was also lower than in mHEL-Tg thymi.

It is noteworthy that low levels of serum HEL were detected in sHEL-Tg mice but not in mHEL-Tg mice (Table I). Although it is

possible that serum HEL contributed to the deletion of T cells in sHEL double-Tg mice, it is unlikely that the effect was substantial; extensive deletion of $1G12^+$ cells was found in the thymus of these mice (Fig. 3*B*), along with intense apoptosis in the medulla (Fig. 7*C*), where the serum HEL is not effective (Fig. 7*E*). Our assumption is also in line with the data of Klein et al. (25) showing that tolerance to a neo-self-Ag is determined mainly by thymic deletion, irrespective of differing serum levels of the Ag.

In addition to deletion of HEL-specific T cells, sHEL double-Tg mice also exhibited a moderate level of T cell anergy (Fig. 6). No lymphocyte anergy was detected in mHEL double-Tg mice (Fig. 6), suggesting that the form of the Ag determines the induction of anergy. This notion is also in line with a similar observation made by Akkaraju et al. (14). It remains to be determined as to whether anergy induction occurs thymically or peripherally in the sHEL double-Tg mice.

Actual deletion of HEL-specific T cells in the thymus was visualized and measured in this study by the TUNEL- and Annexin V-binding assays; it is assumed that the increased apoptosis seen in double-Tg mice is because of programmed death of maturing thymocytes expressing the 3A9 TCR upon their exposure to HEL. Indeed, a good correlation is seen in the present study between the level of elevated apoptosis in thymi of the two lines of double-Tg mice (Fig. 7, *A* and *B*) and the reduction in numbers of cells stained with the 3A9 clonotypic 1G12 Ab (Fig. 3), as well as the decrease in the lymphocyte responsiveness to HEL of these mouse lines (Fig. 4).

The TUNEL assay also provides information concerning the location at which clonal deletion of T cells takes place. In line with observations by Wack et al. (26) and Douek et al. (27), the elevated apoptosis in both lines of double-Tg mice localized mainly in the thymic medulla and the cortex/medulla junction. This observation is also in accord with reports by Kyewski, Hanahan, Pugliese, and their coworkers that medullary cells in thymi of mice (9, 28) or humans (29) express various tissue-specific or neo-self-Ags. It is of note that, unlike in double-Tg mice, the elevated apoptosis in single-Tg 3A9 mice following a bolus injection of HEL localized mainly in the thymic cortex (Fig. 7*E*), a finding in line with the observation by Wack et al. (26). As shown by Martin and Bevan (30), the massive apoptosis in thymic cortex of systemically injected 3A9 mice could be attributed to both Ag-specific deletion of thymocytes and the toxic effect of cytokines released by activated lymphocytes in the periphery. Taken together, these observations indicate that clonal-specific deletion is mediated by HEL synthesized in situ rather than by circulating Ag.

The selective location of the apoptotic process in the thymic medulla is also confirmed by our finding (Fig. 7*A*) that binding of Annexin V, an indicator for dying cells (21), was observed mainly in SP thymocytes, cells that are mostly located in the medulla. The observation that Annexin V binding was also seen in DP thymocytes (Fig. 7*A*) suggests that apoptosis also took place in the deep layers of the cortex.

Analysis of the proliferative response of spleen cells from the different Tg mouse lines further established the deletion of HEL-specific T cells in the double-Tg mice and the superior deletional process in the sHEL-Tg mice. Our data also confirm the notion (2–5) that the negative selection mechanism eliminates mostly the thymocytes that express TCR with high affinity/avidity. The selective deletion was indicated by the skewed response toward higher HEL concentrations in cultures of double-Tg lymphocytes (Fig. 4) and was further verified by our findings using the CFSE dilution technique (Fig. 5*B*) and cultures of mixed cells from Tg and WT mice (Fig. 5*A*). Both assays demonstrated that lymphocytes from double-Tg mice failed to respond mostly against low HEL concentrations.

It is noteworthy that a correlation is seen in this study between the loss of lymphocyte response to low HEL concentrations in culture (Figs. 4 and 5) and the loss of $1G12^{high}$ T cells in the double-Tg mice (Fig. 3*A*), apparently attributable to selective thymic deletion. Assuming that Tg TCR molecules are homogeneous in their secondary and even tertiary structures, we propose, therefore, that the negative selection of thymocytes with Tg TCR deletes selectively cells with high-density TCR in a process analogous to deletion of thymocytes with high affinity in WT animals. It is further assumed that the $1G12^{int}$ thymocytes also express endogenous TCRα.

Double-Tg mice were found in this study to develop ocular inflammation, characterized mainly by cellular accumulation of lymphoid cells, in and around blood vessels (Fig. 1, *B* and *D*), as well as retinal edema in the mHEL double-Tg mice (Fig. 1*D*). It is of note that local accumulation of lymphocytes and mild inflammation were observed by Akkaraju et al. (14) in double-Tg mice expressing HEL in the pancreas or thyroid. Our finding that the pathological changes were more severe in mHEL double-Tg mice (Fig. 1) could be explained by both the larger numbers of HEL-specific T cells in these mice than in the sHEL double-Tg mice and the phenotypic ocular changes only in mHEL-Tg mice. These changes include dystrophy and release of HEL from the lens (Fig. 1*C* and Ref. 10) that facilitate the immunopathological process (31). Additional studies are in progress to define the features characterizing the inflammatory ocular changes and the cells accumulating in the affected mouse eyes.

Experiments with bone marrow chimeras have provided additional useful information concerning the deletion process. In accord with other reports, using various Ags and experimental approaches (23–25, 32), we found that both forms of HEL are expressed by radiation-resistant stromal thymus cells, but not by bone marrow-derived cells (at least not at levels capable of inducing deletion). The involvement of thymic dendritic cells and macrophages in clonal deletion has been controversial (33–35). Several publications have shown that thymic dendritic cells or macrophages do express peripheral Ags (29, 33, 34), but their capacity to induce deletion apparently depends on the cellular location of the Ag. Klein et al. (36) have shown that thymic hemopoietic cells are highly capable of deleting specific Th lymphocytes, but only when the Ag was introduced to the thymus via the blood stream (36). Moreover, data of Oukka et al. (37) indicate that bone marrow-derived thymic cells were incapable of causing deletion even when expressing transgenically the target Ag in their nuclei. This observation thus suggests that bone marrow-derived cells are incapable of presenting intracellular Ag via the MHC class II endogenous pathway.

Since dendritic cells are the most potent APC, it has been suggested and experimentally shown that these cells participate in the thymic deletion process by the mechanism of cross-presentation, i.e., dendritic cells present efficiently Ags produced by stromal epithelium (see Ref. 23). Cross-presentation does not take place in all systems however, as demonstrated in the study of Klein et al. (36). Data reported in the present study may therefore shed new light on this issue by showing the importance of the biophysical properties of the Ag. We thus propose that the superior tolerogenicity of sHEL derives from its being efficiently cross-presented by thymic dendritic cells following release from the medullary epithelial cells. mHEL, on the other hand, is assumed to be presented to the T lymphocytes mainly by the epithelial cells that are less efficient APC than dendritic cells. The capacity of epithelial cells

to autonomously present Ag and induce tolerance was elegantly demonstrated by Oukka et al. (37).

In conclusion, the present study compared for the first time the capacity of soluble and membrane-bound forms of an Ag, HEL, to induce thymic deletion and T cell tolerance. sHEL was found to be superior to mHEL by all parameters employed, testing the actual deletion of HEL-specific T cells as well as its outcome, i.e., the reduction in numbers of HEL-specific T cells and in responsiveness to the Ag. This observation is remarkable in view of the finding that the membrane-bound form of HEL is more effective than the soluble form in inducing tolerance and deletion of B cells. Both forms of HEL are produced by thymic stromal cells and the superiority of sHEL in deleting T cells supports the model in which negative selection achieves high efficiency when the self-Ag is transferred from thymic epithelial cells to the highly potent APC, the bone marrow-derived dendritic cells.

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References

1. Kappler, J. H., M. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell 49:273.*
2. Kisielow, P., and H. von Boehmer. 1995. Development and selection of T-cells: facts and puzzles. *Adv. Immunol. 58:87.*
3. Fowlkes, B. J., and F. Ramsdell. 1993. T-cell tolerance. *Curr. Opin. Immunol. 5:873.*
4. Sprent, J., and H. Kishimoto. 1998. T cell tolerance and the thymus. *Ann. NY Acad. Sci. 841:236.*
5. Stockinger, B. 1999. T lymphocyte tolerance: from thymic deletion to peripheral control mechanisms. *Adv. Immunol. 71:229.*
6. Peterson, D. A., R. J. DiPaolo, O. Kanagawa, and E. R. Unanue. 1999. Quantitative analysis of the T cell repertoire that escapes negative selection. *Immunity 11:453.*
7. Surh, C. D., and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature 372:100.*
8. Klein, L., and B. Kyewski. 2000. Self-antigen presentation by thymic stromal cells: a subtle division of labor. *Curr. Opin. Immunol. 12:179.*
9. Derbinski J., A. Schulte, B. Kyewski, and L. Klein. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol. 2:1032.*
10. Lai, J. C., A. Fukushima, E. F. Wawrousek, M. C. Lobanoff, P. Charukamnoetkanok, S. J. Smith-Gill, B. P. Vistica, R. S. Lee, C. E. Egwuagu, S. M. Whitcup, and I. Gery. 1998. Immunotolerance against a foreign antigen transgenically expressed in the lens. *Invest. Ophthalmol. Vis. Sci. 39:2049.*
11. Ho, W. Y., M. P. Cooke, C. C. Goodnow, and M. M. Davis. 1994. Resting and anergic B-cells are defective in CD28-dependent costimulation of naïve CD^+ T cells. *J. Exp. Med. 179:1539.*
12. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature 334:676.*
13. Hartley, S. B., J. Crosbie, R. Brink, A. B. Kantor, A. Basten, and C. C. Goodnow. 1991. Elimination from the peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature 353:765.*
14. Akkaraju, S., W. Y. Ho, D. Leong, K. Canaan, M. M. Davis, and C. C. Goodnow. 1997. A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis and insulitis. *Immunity 7:255.*
15. Smith-Gill, S. J., A. C. Wilson, M. Potter, R. J. Feldmann, C. R. Mainhart. 1982. Mapping the antigenic epitope for a monoclonal antibody against avian lysozyme. *J. Immunol. 128:314.*
16. Egwuagu, C. E., C. R. Yu, M. Zhang, R. M. Mahdi, S. J. Kim, and I. Gery. 2002. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J. Immunol. 168:3181.*
17. Jiang, S. P., and M. S. Vacchio. 1998. Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft." *J. Immunol. 160:3086.*
18. de Vos, A. F., A. Fukushima, M. C. Lobanoff,. B. P. Vistica, J. C. Lai, J.-C. Grivel, E. F. Wawrousek, S. M. Whitcup, and I. Gery. 2000. Breakdown of tolerance to a neo-self antigen in double transgenic mice in which B cells present the antigen. *J. Immunol. 164:4594.*
19. Bird, J. J, D. R. Brown, A. C. Jullen, N. H. Moskowitz, M. A. Mahowald, J. R. Sider, T. F. Gajewski, C.-R. Wang, and S. L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity 9:229.*
20. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T-cell antigen receptor occupancy. *Annu. Rev. Immunol. 7:445.*
21. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis: flow cytometric detection of phoshatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. *J. Immunol. Methods 184:39.*
22. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol. 119:493.*
23. Viret, C., A. K. Barlow, and C. A. Janeway, Jr. 1999. On the intrathymic intercellular transfer of self-determinants. *Immunol. Today 20:8.*
24. Humblet, C., A. Y. Rudensky, and B. Kyewski. 1994. Presentation and intercellular transfer of self antigen within the thymic microenvironment: expression of the Eα peptide-I-A^b complex by isolated thymic stromal cells. *Int. Immunol. 6:1949.*
25. Klein, L., T. Klein, U. Ruther, and B. Kyewski. 1998. CD4 T cell tolerance to human C-reactive protein, an inducible serum protein, is mediated by medullary thymic epithelium. *J. Exp. Med. 188:5.*
26. Wack, A., H. M. Ladyman, O. Williams, K. Roderick, M. A. Ritter, and D. Kioussis. 1996. Direct visualization of thymocyte apoptosis in neglect, acute and steady-state negative selection. *Int. Immunol. 8:1537.*
27. Douek, D. C., K. T. T. Corley, T. Zal, A. Mellor, P. J. Dyson, and D. Altmann. 1996. Negative selection by endogenous antigen and superantigen occurs at multiple thymic sites. *Int. Immunol. 8:1413.*
28. Smith, K. M., D. C. Olson, R. Hirose, and D. Hanahan. 1997. Pancreatic gene expression in rare cells of thymic medulla; evidence for functional contribution to T cell tolerance. *Int. Immunol. 9:1355.*
29. Pugliese, A., D. Brown, D. Garza, D. Murchison, M. Zeller, M. Redondo, J. Diez, J. S. Eisenbarth, D. D. Patel, and C. Ricordi. 2001. Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J. Clin. Invest. 107:555.*
30. Martin, S., and M. J. Bevan. 1997. Antigen-specific and nonspecific deletion of immature cortical thymocytes caused by antigen injection. *Eur. J. Immunol. 27: 2726.*
31. Lai, J. C., M. C. Lobanoff, A. Fukushima, E. F. Wawrousek, C.-C. Chan, S. M. Whitcup, and I. Gery. 1999. Uveitis induced by lymphocytes sensitized against a transgenically expressed lens protein. *Invest. Ophthalmol. Vis. Sci. 40: 2735.*
32. Zinkernagel, R. M., and A. Althage. 1999. On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells. *Proc. Natl. Acad. Sci. USA 96:8092.*
33. Pietropaolo, M., N. Giannoukakis, and M. Trucco. 2002. Cellular environment and freedom of gene expression. *Nat. Immunol. 3:335.*
34. Pugliese, A., and J. Diez. 2002. Lymphoid organs contain diverse cells expressing self-molecules. *Nat. Immunol. 3:335.*
35. Kyewski, B., J. Derbinski, A. Schulte, and L. Klein. 2002. Response. *Nat. Immunol. 3:336.*
36. Klein, L., B. Roettinger, and B. Kyewski. 2001. Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. *Eur. J. Immunol. 31:2476.*
37. Oukka, M., E. Colucci-Guyon, P. L. Tran, M. Cohen-Tannoudji, C. Babinet, V. Lotteau, and K. Kosmatopoulos. 1996. CD4 T cell tolerance to nuclear proteins induced by medullary thymic epithelium. *Immunity 4:545.*