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Mouse Lysozyme-M Knockout Mice Reveal How the Self-Determinant Hierarchy Shapes the T Cell Repertoire against This Circulating Self Antigen in Wild-Type Mice[1]

Pratima Sinha,* Howard H. Chi,* Hong R. Kim,* Björn E. Clausen,† Brian Pederson,‡ Eli E. Sercarz,‡§ Irmgard Forster,¶ and Kamal D. Moudgil[2]*

We have studied T cell tolerance to defined determinants within ML-M using wild-type (WT; ML-M$^{+/+}$) and LysMcre (ML-M$^{-/-}$) C3H (H-2^{k}) mice to determine the relative contribution of ML-M-derived epitopes vs those from other self Ags in selection of the ML-M-specific T cell repertoire. ML-M was totally nonimmunogenic in WT mice, but was rendered immunogenic in LysMcre mice. Most of the response to ML-M in LysMcre mice was directed to the immunodominant determinant region 105–119. This determinant is spontaneously displayed (without adding exogenous ML-M) by macrophages of WT, but not LysMcre, mice and is stimulatory for peptide 105–119 (p105–119)-primed T cells. Moreover, neonatal tolerization of LysMcre mice with p105–119 or ML-M abrogated the T cell response to subsequent challenge with ML-M or p105–119. Furthermore, p95–109 and p110–125 of ML-M were immunogenic in LysMcre mice, but not in WT mice, thereby representing subdominant, tolerance-inducing epitopes of ML-M. As expected, the T cell repertoire to cryptic ML determinants in WT mice was also intact in LysMcre mice. Furthermore, the pattern of response to the related homologue of ML-M, hen eggwhite lysozyme, was similar in these two groups of mice. Thus, several codominant T cell determinants within ML-M contribute significantly to tolerance induction, and the anti-cryptic T cell repertoire to ML-M was positively selected on non-ML-M self ligands. These results reveal that the induction of self tolerance to a multideterminant protein follows the quantitative hierarchy of self-determinant expression and are of relevance in understanding the pathogenesis of autoimmunity. ***The Journal of Immunology*****, 2004, 173: 1763–1771.**

The T cell repertoire is shaped in the thymus through complex processes of positive and negative selection. T cells bearing receptors that interact with self MHC-self peptide complexes with a moderate to low avidity get positively selected, whereas T cells possessing TCR that either have a strong reactivity with self MHC-peptide (potentially autoreactive) or no reactivity at all (ignorance for self MHC) get negatively selected through clonal deletion and/or anergy (central tolerance) or are bypassed by selective forces and die of neglect (1–6). Similarly, a diverse set of regulatory mechanisms has been invoked to control T cell reactivity in the periphery (peripheral tolerance) (7–10). Effective induction of self tolerance, both central and peripheral, is crucial for prevention of autoimmunity.

Most of the available information on negative selection of CD4^{+} T cells is based on transgenic mice in which a neo-self Ag is expressed either as a secreted protein or as a membrane-bound/cytosolic Ag under a ubiquitous or tissue-specific promoter (11–16). It is conceivable that regulation of the level of such a neo-self Ag in blood and other tissues might be quite different from that of a naturally occurring self protein such as mouse lysozyme-M (ML-M)[3] (17, 18), which is synthesized and secreted by cells of the myeloid lineage. Although these studies in transgenic mice have provided interesting insights into self tolerance, it is not clear how much of this information is applicable in the natural, physiologic settings of a circulating or tissue-specific self Ag. Another set of studies based on mice deficient in a specific self Ag, either naturally or experimentally (Ag knockout mice), have further enhanced our understanding of the impact of self Ag on shaping of the T cell repertoire (19–25). However, some critical aspects of epitope-specific tolerance described below have either not been addressed or not fully examined in these model systems.

To fully understand the in vivo shaping of the T cell repertoire directed against a given self Ag (for example, ML-M) (17, 18) as well as tolerance to that Ag, it is essential to define the relative contribution to these processes of the self determinants displayed by the APCs in the thymus (or in the periphery) after processing of the same self Ag compared with that of another set of self peptides originating from other self Ags. For positive selection, some evidence suggests that even a very few peptides can select a broad T cell repertoire (4, 26–28). However, there is not much information regarding either the specificity or the diversity of negatively selecting peptides. In this context, what is the contribution of T cell determinants within ML-M vis-à-vis self determinants on other proteins in shaping of the self (ML-M)-directed T cell repertoire?

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[3] Abbreviations used in this paper: ML-M, mouse lysozyme-M; DC, dendritic cell; HEL, hen eggwhite lysozyme; LNC, lymph node cell; LysMcre, mice lacking ML-M; PPD, purified protein derivative; WT, wild type; SI, stimulation index.

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Furthermore, is the presence of ML-M critical in positively selecting the T cells potentially specific for various determinants (dominant/cryptic) within this self protein? Considering that up-regulation of the display of previously cryptic epitopes is involved in the induction/propagation of autoimmunity (29–32) and graft rejection (33), and that the anti-cryptic T cell repertoire can be harnessed for induction of antitumor immunity (34–36), it is important to examine the thymic selection of the T cell repertoire specific for cryptic self determinants. Another related question is the involvement of ML-M, if any, in positive or negative selection of the T cell repertoire for its related homologue, hen eggwhite lysozyme (HEL), either in closely parallel regions of the molecule or in areas of quite distinct amino acid sequence. Examination of the interrelationship between ML-M and HEL is prompted by our earlier findings in wild-type (WT) mice showing that the T cells primed by certain peptides of ML-M representing cryptic self determinants could be efficiently recalled by native HEL (18) and vice versa (37), suggesting that subsets of the T cell repertoire might be shared between these two proteins.

To address these important questions, we have studied the T cell repertoire to defined determinants within the circulating form of mouse lysozyme, ML-M, in both WT C3H (H-2^k; ML-M-sufficient (ML-$M^{+/+}$)) mice and LysMcre C3H (ML-M-deficient (ML-$M^{-/-}$)) mice (in which the ML-M gene was mutated by insertion of cre-cDNA into the first exon of the ML-M gene (38). We observed that ML-M, which was totally nonimmunogenic in WT C3H mice, is rendered immunogenic in LysMcre mice. Most of the response to ML-M in LysMcre was accounted for by the dominant epitope within the region 105–119. Our results demonstrate that both the dominant and subdominant T cell determinants within ML-M contribute significantly to negative selection of the potentially autoreactive T cell repertoire, and that the anti-cryptic T cell repertoire against ML-M was positively selected on non-ML-M self ligands. Furthermore, there was no significant effect on the T cell response to dominant determinants of the homologous HEL of the absence of ML-M.

Materials and Methods

Mice

WT C3H (C3H/HeJ; H-2^k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility at University of Maryland School of Medicine (Baltimore, MD). Male mice, 2–4 mo old, were used in the experiments. All procedures performed on these mice followed the guidelines of the institutional animal care and use committee.

Generation of ML-M knockout (LysMcre) C3H mice

The original LysMcre mice were generated in the laboratory of one of us (38) using the bacteriophage P1-derived Cre/loxP recombination system, expressing Cre in myeloid cells after targeted insertion of the cre cDNA into their endogenous M lysozyme locus (38). One line of female founder LysMcre (H-2^k) mice was backcrossed for a total of 10 generations onto the C3H (H-2^k) background using C3HeB/FeJ mice. At the 10th generation, intercrossing between heterozygous (ML-$M^{+/-}$) LysMcre mice was performed to generate homozygous (ML-$M^{-/-}$) LysMcre mice. Male mice, 2–4 mo of age, were used in this study.

PCR genotyping of LysMcre mice

All mice were screened by PCR to distinguish between the WT mice and mice carrying the Cre-inserted ML-M allele (heterozygous or homozygous). For this purpose, the tail samples (0.5 cm of a distal tail piece) from mice were digested by incubation at 55°C overnight with proteinase K (Invitrogen Life Technologies, Grand Island, NY). The subsequent phenol-chloroform extraction yielded DNA templates for PCR. Each DNA sample was then amplified by PCR in a reaction mixture of 1 mM dNTPs, *Taq* polymerase buffer with 6.25 mM $MgCl_2$, and *Taq* polymerase using a set of three primers (38): Cre8, 5′-CCCAgAAATgCCAgATTACg-3′; Mlys1, 5′-CTTgggCTgCCAgAATTTCTC-3′; and Mlys2, 5′-TTACAgTCggC CAggCTgAC-3′ (Sigma Genosys, The Woodlands, TX). All samples were subjected serially to the following reaction cycles using a 96-well thermal cycler (MJ Research, Watertown, MA); 1) 94°C for 2.5 min to denature DNA complexes; 2) 36 cycles, each consisting of the following sequential steps: 1 min at 94°C, 1 min at 63°C, and 1.5 min at 72°C; and 3) 10 min at 72°C. These PCR products were analyzed by 1.5% agarose gel electrophoresis, and the resulting band patterns were used to determine the genotype of the screened animals.

Lyso-plate bioassay for measuring the level of ML-M in serum

Lysozyme activity was measured in sera using the standard agar diffusion assay (39) involving the lysis of *Micrococcus lysodeikticus* cells in agar. After 48-h incubation at room temperature, the plates were scanned using an Alpha Imager (Alpha Innotech, Mississauga, Canada), and the diameter of the lytic zone resulting from bacterial lysis was measured. HEL and ML-M were used as standards. Each sample was tested in duplicate, and the test was repeated.

Analysis of lymphoid cells by flow cytometry

Blood (0.5 ml) from the mice was collected in heparinized tubes, then subjected first to lysis of RBC, followed by treatment of cells with FcγR-blocking Ab (secreted by the B cell line 2.4 G2 (American Type Culture Collection, Manassas, VA)) to eliminate nonspecific binding. Thereafter, the cells (1×10^6) were stained with the appropriate Abs, including isotype controls (PE-conjugated anti-mouse I-A^b (clone AF6-120.1), FITC-anti-mouse I-A^k (clone 11-5.2), FITC-anti-mouse I-A^d/E^d (clone AMS-32.1), PE-mouse IgG2a (clone G155-178), and FITC-mouse IgG2b (clone 49.2); purchased from BD Pharmingen, San Diego, CA). Finally, the stained cells were fixed in 0.5 ml of 1% paraformaldehyde, and 20,000 events were acquired in a FACSort flow cytometer (BD Biosciences, Sunnyvale, CA). The data analysis was performed using CellQuest software (BD Biosciences).

Ags/mitogens

Synthetic peptides containing sequences of ML-M and HEL were obtained from Macromolecular Resources and Global Peptide Services (both in Fort Collins, CO). The amino acid residue (glycine) at position 48 of ML-M (130 aa) has been numbered 47a for accurate comparison of position of epitopes of ML-M and HEL (129 aa) (18). The relative binding activities of different ML-M peptides to the A^k/E^k molecules have been described previously (37) (data not shown). HEL, keyhole limpet hemocyanin, and Con A were purchased from Sigma-Aldrich (St. Louis, MO), and HEL was further purified by ion exchange chromatography. Tuberculin-derived purified protein derivative (PPD) was obtained from Parke-Davis (Morris Plains, NJ).

Preparation of ML-M

ML-M is secreted by the mouse macrophage cell line J774 A.1 (American Type Culture Collection) that synthesizes this protein. This cell line was maintained in culture and then grown as a tumor in the peritoneal cavity of BALB/c mice as described previously (18). The ML-M in ascitic fluid was then purified by ion exchange chromatography using carboxymethyl cellulose (CM 52; Whatman, Maidstone, U.K.) using a modification of the method described previously (18). The purity of ML-M was checked by matrix-assisted laser desorption ionization using Voyager Biospectrometry (PerSeptive Biosystems, Framingham, MA) in the biopolymer core facility at our institution (University of Maryland School of Medicine). The identity of ML-M was further validated by sequence analysis of the first eight N-terminal amino acid residues performed at the Howard Hughes Medical Institute biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Lymph node cell (LNC)/spleen cell (SPC) proliferation assay

Groups of four to six mice were immunized s.c. at the base of the tail either with native ML-M (7 nmol/mouse; unless indicated otherwise) or with a peptide of ML-M (33 nmol/mouse) in PBS, each in a 1/1 (v/v) emulsion with CFA (Difco, Detroit, MI). After 9 days, the draining LNC/SPC were tested in a proliferation assay (37), typically using native ML-M (3.8 μM, final concentration) or a peptide of ML-M (33 μM, final concentration) for in vitro recall response. (The dose of Ag used for immunization or in vitro recall was found to be optimal in pilot experiments. HEL or its peptides were also tested at the same dose as that used for ML-M or its peptides.) The results were expressed as a stimulation index (SI = cpm with Ag/cpm without Ag; mean ± SEM).

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Proliferation assay using purified Ag-primed T cells and APC

T cell enrichment from LNC. LysMcre (H-2^k) mice were immunized with a peptide of ML-M emulsified in CFA, and on the ninth day thereafter, LNC were harvested. These LNC were labeled with MACS colloidal superparamagnetic microbeads conjugated to the appropriate Ab (Miltenyi Biotech, Auburn, CA) and then processed using an autoMACS separator to obtain T cell enriched fraction of cells. In some experiments, the T cells were purified from LNC using a nylon wool column (40).

Preparation of APC (macrophages). One milliliter of 1% thioglycolate broth (Difco) in water was injected i.p. into mice, and 3 days later the peritoneal macrophages were collected in PBS (41).

T cell proliferation assay. ML-M peptide-primed enriched T cells were cultured with APCs from WT or LysMcre mice, with or without Ag. Proliferation of cells was measured as described previously (37).

Neonatal tolerance induction

Neonatal LysMcre mice were tolerized with ML-M or p105–119 of ML-M (each emulsified in IFA) using a method described previously (42). A total of two injections were given i.p. to the neonate: one within the first 18 h of life and another at 72 h (each injection consisting of 50 μl of emulsion containing a total of 1.75 nmol of protein or 8.25 nmol of peptide). A control group of neonates was given an i.p. injection of PBS/IFA. These mice were then used in experiments at the age of 4 wk.

Results

Phenotypic and genotypic characterization of the LysMcre C3H (ML-$M^{-/-}$) mice

LysMcre mice were generated as described in *Materials and Methods* (38). Before using them for immunologic studies, we characterized their homozygous/heterozygous status for the ML-M allele (by PCR analysis), for the presence or absence of native ML-M in blood (by bioassay for ML-M activity), and for their MHC haplotype (by flow cytometry). The results of these experiments are given in Fig. 1. Fig. 1*A* depicts the representative PCR profiles of WT (ML-$M^{+/+}$), heterozygous (ML-$M^{+/-}$) LysMcre, and homozygous (ML-$M^{-/-}$) LysMcre mice. The PCR reveals a 350-bp band in WT C3H mice, whereas 700-bp and 1.7-kbp bands appear in LysMcre (ML-$M^{-/-}$) mice; all three (350 bp, 700 bp, and 1.7 kbp) are amplified in LysMcre (ML-$M^{+/-}$) mice. Fig. 1*B* demonstrates the absence of ML-M in the blood of homozygous LysMcre (ML-$M^{-/-}$) mice, and Fig. 1*C* shows that the LysMcre line of mice is of the H-2^k haplotype. Thus, the LysMcre mice are functional knockouts for self lysozyme, ML-M.

T cell response to native ML-M of LysMcre (ML-$M^{-/-}$) and WT (ML-$M^{+/+}$) C3H mice

To determine the influence of the absence of ML-M on the in vivo T cell response to native ML-M or to various determinants within ML-M, we tested the responses to native ML-M of LysMcre (ML-M deficient) and compared the response to that of WT (ML-M sufficient) mice. Mice were challenged s.c. with native ML-M emulsified in CFA, and after 9 days, the draining LNC were tested in a proliferation assay using peptides of ML-M as well as native ML-M for in vitro recall response. The results given in Fig. 2 show that LysMcre, but not WT C3H, mice raise a T cell response to both native ML-M and p105–119 of ML-M. Furthermore, the induction as well as recall of the T cell response to ML-M and its peptide 105–119 was dose dependent (Fig. 3). Similarly, heterozygous (ML-$M^{+/-}$) mice behaved like WT mice (data not shown); both were tolerant to native ML-M. Thus, there was a direct and significant correlation between the presence (or the absence) of ML-M in the circulation and induction of T cell tolerance (or lack of tolerance), respectively, to the same Ag.

Neonatal tolerization of LysMcre mice to p105–119 of ML-M abrogates response to native ML-M, and vice versa

We tolerized the LysMcre mice with ML-M p105–119 in the neonatal period and then tested their T cell response after immunization with either p105–119 or ML-M at 4 wk of age to determine whether they became unresponsive to ML-M like WT mice. Fig. 4 shows that the neonatally tolerized LysMcre mice failed to raise a T cell response to p105–119 or ML-M. As expected, PBS-tolerized LysMcre gave a strong response to the same immunogens. Similar results were obtained from LysMcre mice neonatally tolerized against native ML-M: a complete loss of response to both ML-M and p105–119 (data not shown). Thus, LysMcre mice neonatally tolerized with p105–119 or ML-M behaved like WT mice. These results again demonstrate that the T cell response to determinant region 105–119 comprises a majority of the response of LysMcre mice to native ML-M.

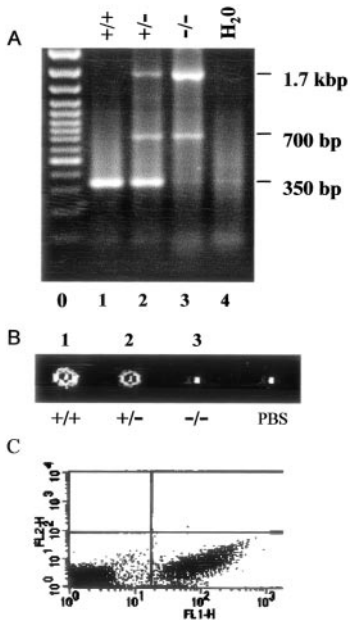

FIGURE 1. Characterization of LysMcre (ML-$M^{-/-}$) C3H (H-2^k) mice. *A*, PCR analysis of genomic DNA prepared from mouse tail samples of LysMcre mice. Genomic DNA was PCR-amplified, then analyzed in a 1.5% agarose gel. *Lane 0*, Ladder DNA, 1 kb; *lane 1*, WT C3H (ML-$M^{+/+}$) mice; *lane 2*, heterozygous (ML-$M^{+/-}$) LysMcre mice; *lane 3*, homozygous (ML-$M^{-/-}$) LysMcre mice; *lane 4*, water control. The PCR amplifies a 350-bp band in WT C3H mice, whereas 700-bp and 1.7-kbp bands appear in homozygous LysMcre mice; however, in heterozygous LysMcre mice all three (350 bp, 700 bp, and 1.7 kbp) are amplified. *B*, Bioassay to measure the level of ML-M in mouse serum. *Lane 1*, WT C3H (ML-$M^{+/+}$) mice; *lane 2*, heterozygous (ML-$M^{+/-}$) LysMcre mice; *lane 3*, homozygous (ML-$M^{-/-}$) LysMcre mice; and PBS control. *C*, Flow cytometric analysis of PBMC of LysMcre mice for the MHC (H-2^k) haplotype using the FITC-anti-I-A^k Ab (FL1; *x*-axis) and PE-anti-I-A^b/I-A^d (control) Ab (FL2; *y*-axis).

Spontaneous display of potentially dominant self (ML-M) determinant on APCs of WT mice

To determine whether the epitope region 105–119, which represents a dominant determinant within exogenously administered ML-M, is also naturally processed and presented from endogenous ML-M, we tested in vitro restimulation of p105–119-primed T cells of LysMcre mice by macrophages (that synthesize and secrete ML-M) from age- and sex-matched, naive WT C3H or naive LysMcre mice without addition of any exogenous ML-M/p105–119. The T cells primed by p1–15 containing a cryptic epitope of ML-M (described below) served as controls for p105–119-primed T cells. The results given in Fig. 5 clearly demonstrate that p105–119-primed, but not p1–15-primed, T cells cultured with WT macrophages gave a

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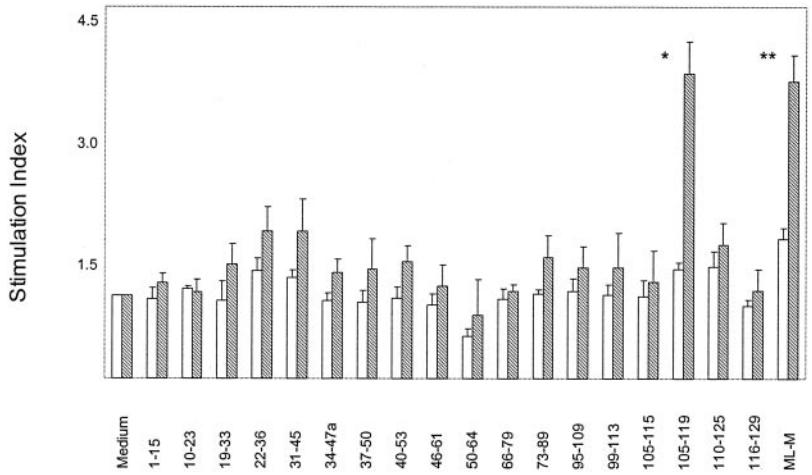

FIGURE 2. Response to peptides of ML-M of WT C3H (ML-$M^{+/+}$; □) and LysMcre C3H (ML-$M^{-/-}$; ▨) mice challenged with native ML-M. Mice (n = 5/group) were immunized s.c. at the base of the tail with ML-M (7 nmol/mouse) emulsified in CFA, and on the ninth day, the response of LNC was recalled in vitro with native ML-M (3.8 μM, final concentration) or with peptides (33 μM, final concentration each) covering the entire length of ML-M. (The dose of Ags used in this and other experiments was found to be optimal in pilot experiments. Unless stated otherwise, the dose for challenge of mice and for in vitro recall of response with native protein/peptide shown in other figures is the same as that given in this figure.) The results are shown as mean SI units ± SEM. The response to PPD had SI values of 44 ± 11 and 79 ± 19 in WT C3H and LysMcre mice, respectively. *, $p < 0.0005$; **, $p = 0.005$.

significantly higher proliferative response compared with those cultured with LysMcre macrophages. These results demonstrate that the determinant region 105–119 of ML-M was spontaneously displayed (presumably as a peptide-MHC complex) on WT macrophages, but not on LysMcre macrophages, and it represents a naturally processed determinant within endogenous ML-M. On the contrary, as expected, the cryptic epitope 1–15 failed to be effectively presented from endogenous ML-M.

Response to potentially immunogenic, MHC (A^k/E^k) binding ML-M peptides of LysMcre (ML-$M^{-/-}$) mice

A multideterminant self Ag such as ML-M is likely to have three types of T cell determinants based on their MHC binding and immunogenicity in WT (ML-$M^{+/+}$) mice: 1) those that bind MHC, but are nonimmunogenic (potentially tolerogenic, dominant determinants); 2) those that bind to MHC and are immunogenic (cryptic determinants); and 3) those that do not bind MHC and are nonimmunogenic (nondeterminants). In view of the above-mentioned observation of response to only one ML-M determinant region, 105–119, of LysMcre mice after immunization with native ML-M (Fig. 2), we tested whether the pattern of T cell response to four ML-M peptides that bind to A^k/E^k molecules and thereby are potentially immunogenic was influenced by the absence of ML-M in LysMcre (ML-$M^{-/-}$) mice compared with WT mice. For this purpose, mice were immunized with a peptide of ML-M in CFA, and 9 days thereafter the response of LNC of these mice was tested in vitro with the same ML-M peptide or with native ML-M. Fig. 6 shows that of four peptides (peptides 34–47a, 95–109, 105–119, and 110–125) tested, three peptides (peptides 95–109, 105–119, and 110–125) induced a T cell response in LysMcre mice, but none of these peptides elicited any response in WT mice. The remaining peptide, p34–47a, was nonimmunogenic in both LysMcre and WT mice (data not shown). Furthermore, there was no cross-reactivity among any of the above four peptides tested (data not shown). These results suggest that peptides 95–109, 105–119, and 110–125 represent well-processed and presented codominant epitopes of ML-M, and thereby, the T cell repertoire potentially specific for these determinants was deleted during thymic development in WT mice. However, considering that a majority of the T cell response of ML-M-immunized LysMcre mice was directed to determinant region 105–119, both 95–109 and 110–125 can be categorized as subdominant determinants compared with the dominant epitope 105–119.

Study of the T cell repertoire directed against cryptic determinants within ML-M in WT and LysMcre mice

To define the role of self lysozyme (ML-M) in selection of the T cell repertoire specific for cryptic epitopes within this Ag, WT and

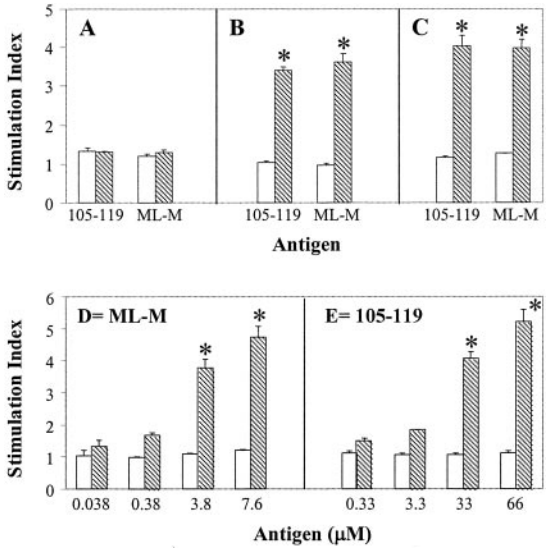

FIGURE 3. Response to ML-M of WT (ML-$M^{+/+}$) (□) and LysMcre C3H (ML-$M^{-/-}$; ▨) mice. *Top panel*, Mice were immunized with ML-M (emulsified in CFA) using a dose of 0.9 (*A*), 2.6 (*B*), or 7.0 (*C*) nmol/mouse. On the ninth day, the draining LNC of these mice were tested in a proliferation assay using peptide 105–119 of ML-M (33 μM, final concentration) or native ML-M (3.8 μM, final concentration) for in vitro recall response. The results (n = 6 each) are shown as a stimulation index (mean ± SEM). *, $p < 0.01$. The responses to PPD of these three groups of mice were comparable. *Bottom panel*, The response of LNC of mice immunized with ML-M (7 nmol/mouse) in CFA was tested in vitro using different recall doses of native ML-M (*D*) or peptide 105–119 of ML-M (*E*). The results (n = 6) are presented as in the *top panel*. *, $p < 0.05$.

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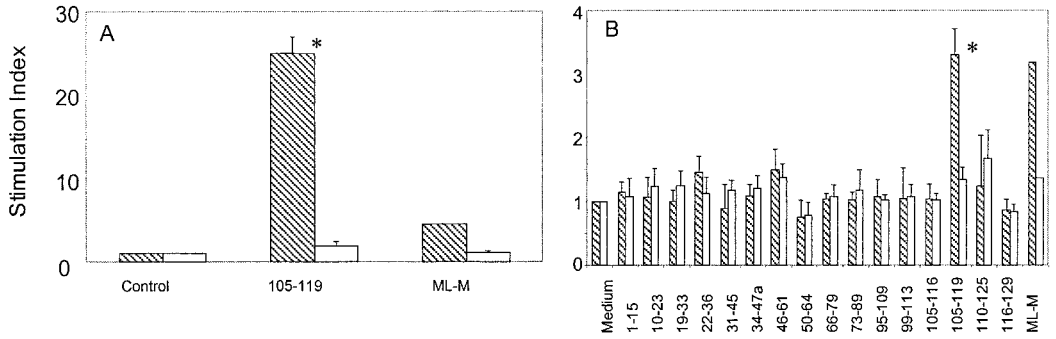

FIGURE 4. Neonatal tolerization of LysMcre (ML-M$^{-/-}$) with PBS/IFA (▨) or p105–119/IFA (□), followed by challenge with either p105–119 (*A*) or native ML-M (*B*) at 4 wk age. Mice were tolerized with ML-M p105–119/IFA or PBS/IFA twice, within 24 and 72 h of birth. At 4 wk of age, both groups of tolerized mice were immunized at the base of the tail with p105–119/CFA (*A*) or ML-M/CFA (*B*), and on the ninth day, the response of LNC was recalled in vitro with native ML-M or a peptide of ML-M. The results ($n = 9$) are shown as mean SI units ± SEM. The response to PPD had SI values of 31 ± 10 to 59 ± 8. *, $p < 0.05$.

LysMcre mice were immunized with MHC binding and potentially immunogenic ML-M peptides (peptides 1–15, 50–64, and 99–113) in CFA, and on the ninth day a proliferation assay was performed using the draining LNC of these mice. (WT mice immunized with ML-M do not raise a T cell response to these three peptides. However, each of these three peptides is immunogenic in WT mice and T cells primed by these peptides are not recalled by native ML-M, demonstrating that these peptides contain cryptic epitopes of ML-M (data not shown).) Results given in Fig. 7 show that both LysMcre mice and WT mice gave a comparable level of response to each of these peptides, with p50–64 giving the highest response, followed by p99–113 and p1–15 in that order. These results indicate that the T cell repertoire to these cryptic determinants was intact in LysMcre mice lacking ML-M, and that it was selected on non-ML-M self ligands. Two other peptides of ML-M, p31–45 and p73–89, which do not bind to MHC and are nonimmunogenic in WT mice, were also found to be nonimmunogenic in LysMcre mice (data not shown). These peptides served as reliable negative controls in this study.

Influence of the absence of ML-M in LysMcre mice on the T cell response of these mice to the homologous lysozyme, HEL

Our preliminary experiments showed the absence of any significant cross-reactivity between native ML-M and HEL; immunization of both WT and LysMcre mice with native ML-M (7 nmol/mouse) failed to induce a significant recall response to HEL (data not shown). However, in our previous work we observed that T cells reactive against certain cryptic epitopes of ML-M were cross-reactive with native HEL and vice versa, suggesting sharing of the T cell repertoire between ML-M and HEL. To determine whether the presence or the absence of native ML-M had any significant influence on the T cell response to defined epitopes within a related homologue of ML, HEL, we tested the T cell response to HEL of LysMcre and C3H WT mice. Mice were immunized with native HEL in CFA, and on the ninth day LNC of these mice were tested in a proliferation assay using HEL or peptides of HEL for in vitro recall response. The results shown in Fig. 8 revealed that there was no significant difference in response to native HEL or to peptides (peptides 1–17, 20–35, 30–53, 46–61, 50–64, and 116–129) comprising the dominant epitopes of HEL in WT and LysMcre mice. Thus, the pattern of response to HEL in LysMcre mice was not influenced significantly by the absence of ML-M compared with that in ML-sufficient WT mice. However, the fine clonal specificity of TCR usage in response to a particular determinant of HEL in WT vs LysMcre mice remains to be determined.

Discussion

In this study we have analyzed the T cell epitope-specific tolerance to ML-M, a circulating self-protein, in its natural physiological setting using ML-M-sufficient (ML-M$^{+/+}$) WT C3H (H-2^k) mice and ML-M deficient (ML-M$^{-/-}$) LysMcre mice. In WT mice, ML-M is synthesized and secreted by cells of the myeloid lineage, e.g., macrophages, granulocytes, and a subset of dendritic cells (DC) that are closely related to the monocyte/macrophage lineage (17, 38). The mean blood level of ML-M has been reported to range from 1.8–2.9 μg/ml (43, 44). ML-M activity is found in various tissues, mostly due to tissue-resident macrophages, with the highest activity reported for kidney, spleen, and lung (17). In comparison, thymus and small intestine revealed moderate levels of ML-M activity. Unlike ML-M, mice transgenic for HEL express

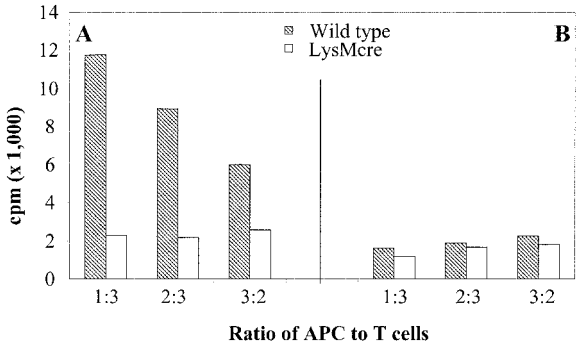

FIGURE 5. Spontaneous display of potentially dominant self (ML-M) determinants on APCs of WT mice. Peritoneal macrophages were collected on the fourth day after sodium thioglycolate injection of WT C3H (▨) or LysMcre (□) C3H mice and used as APC. Purified T cells from the draining LNC of LysMcre mice challenged s.c. 8 days earlier with either p105–119 (*A*) or p1–15 (*B*) of ML-M in CFA were tested in a proliferation assay using WT APC and T cells as the experimental group and LysMcre APC and T cells as the control group, without any addition of exogenous Ag. The APC and T cells were tested at different ratios, e.g., 1.25×10^5/well APC and 3.75×10^5/well T cells (1:3). The results are expressed as Δ cpm. (In both the WT and LysMcre groups, APC and T cells in the presence of p105–119/p1–15 served as positive controls. APC only and T cell only with Ag (p105–119 or p1–15) served as negative controls.) The difference in the average cpm (mean ± SD) obtained using three different ratios of WT APC:T cells compared with average cpm of the corresponding LysMcre APC:T cell ratios was statistically significant ($p < 0.05$, by Student's *t* test) for p105–119-primed T cells (A), but not p1–15-primed T cells (*B*).

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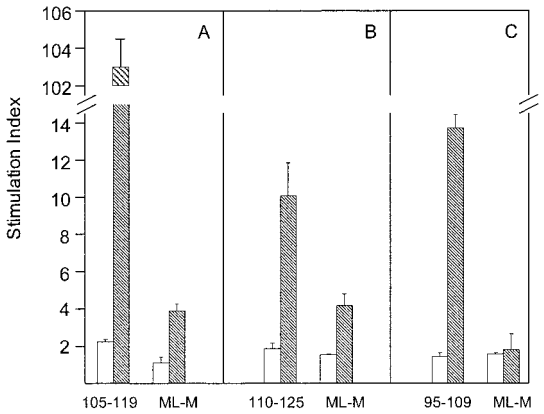

FIGURE 6. The T cell response to selected peptides of ML-M in WT C3H (□) and LysMcre C3H (▨) mice after peptide immunization. Mice (n = 4–6/group) were challenged at the tail base with a peptide of ML-M: p105–119 (*A*), p110–125 (*B*), or p95–109 (*C*), each emulsified in CFA. On the ninth day, the response of the draining LNC was recalled in vitro with the immunizing peptide as well as native ML-M. The response shown represents the results from four to six mice (mean SI units ± SEM). The SI value for PPD in relation to these peptides ranged from 24–73.

this neo-self Ag either as a secreted protein (under the control of a ubiquitous or tissue-specific promoter) or as a membrane-bound protein (11–13, 45). The blood level of HEL in different lines of transgenic mice ranges from 1000 to 2 (or less) ng/ml (11, 46), 43–100 ng/ml (3–7 nM) (12), 9–23 ng/ml (13), and 3 ng/ml (45). Thus, both the patterns of tissue expression (dependent on the type of promoter used for expression of the transgene) as well as the blood level of HEL in mice transgenic for this protein are quite different from those of ML-M in normal (WT) mice. For these reasons, the results of our study based on LysMcre and WT mice provide important information regarding the induction of epitope-specific self tolerance to ML-M in WT mice under physiological conditions.

We observed that WT C3H ($H2^k$) mice were tolerant to native ML-M, whereas LysMcre ($H\text{-}2^k$) mice raised T cell responses to this self Ag after s.c. immunization with ML-M in CFA. Similar results were obtained using ($H\text{-}2^b \times H\text{-}2^d$) LysMcre and the corresponding WT mice (data not shown). The novel feature of this study is the demonstration that the T cell repertoire to a self protein, ML-M, is shaped in the natural physiological setting by the dominance/crypticity of its own determinants; during development of the ML-M-directed T cell repertoire, both dominant and subdominant determinants within ML-M lead to tolerance induction by negative selection in the thymus, whereas crypticity of other determinants allows T cells potentially reactive to these determinants to escape tolerance induction, permitting these T cells to appear in the periphery. Peptides 95–109, 105–119, and 110–125 of ML-M were found to be immunogenic in LysMcre mice, but not in WT C3H mice. These peptides represent the tolerogenic determinants within ML-M. Furthermore, there is a hierarchy among the above three peptides, with 105–119 being the most dominant, followed by the subdominant determinants 95–109 and 110–125. (Peptides 95–109, 105–119, and 110–125 bind to the E^k molecule with decreasing order of binding avidity; of these, only p110–125 binds to the A^k molecule, and this binding is of lower avidity than that for the E^k molecule.) In contrast, peptides 1–15, 50–64, and 99–113 of ML-M represent those epitopes of ML-M that are cryptic for both WT C3H and LysMcre mice. The remaining peptides of ML-M tested were nonimmunogenic in both WT and LysMcre mice. The nonimmunogenic peptides of ML-M were tested at the same concentration as other ML-M peptides that were immunogenic, and the optimal dose range selected for their screening was based on our earlier studies on peptides of self and foreign lysozyme (18, 37, 47). However, it is possible that a peptide of ML-M might be required in exceedingly higher amounts for priming of T cells compared with others. Alternatively, nonimmunogenicity of an ML-M peptide could be due to the nonavailability of the appropriate TCR-bearing T cells in the repertoire (48).

In LysMcre mice, ML-M behaves like a foreign protein. These results are in accord with the findings regarding response to C5 in C5-deficient, but not C5-sufficient, mice (20); responsiveness of male, but not female, mice to an ovarian Ag, ZP3 (21); deletion of H-Y-specific T cells in male, but not female, mice (19); response to mouse MBP in shiverer ($MBP^{-/-}$), but not (or markedly decreased in) WT ($MBP^{+/+}$), mice (22–24); and enhanced response to interphotoreceptor retinoid-binding protein in mice lacking this protein compared with WT mice (25). Study of the T cell response to MBP in C3H ($H\text{-}2^k$) shiverer ($MBP^{-/-}$) vs WT C3H mice revealed that high avidity T cells against the dominant epitope 79–87 of MBP were deleted in WT C3H mice, and that there was a reversal of hierarchy of this epitope from dominant in $MBP^{-/-}$ mice to cryptic in WT mice (22). In B10.PL ($H\text{-}2^u$) shiverer mice, differential tolerance to epitope 121–151, but not 1–11/1–20, was observed in syngeneic WT mice (23). Furthermore, epitope 1–11/1–20 was dominant in WT mice, but was subdominant in $MBP^{-/-}$ mice. In contrast to these studies, in our study the epitope 105–119 of ML-M was dominant in ML-M-immunized LysMcre mice, but no response to this determinant was induced in WT mice. In addition, our study has contributed the following novel aspects of induction of epitope-specific tolerance that have either not been addressed or not fully examined in the above-mentioned studies: 1) elicitation of the T cell repertoire to defined cryptic epitopes of ML-M in LysMcre vs WT mice, showing that this repertoire was selected on non-ML-M self ligands; 2) demonstration that the immunodominant epitope region 105–119 was naturally processed and presented (to specific T cells) from endogenous ML-M by APC of WT, but not LysMcre, mice; 3) validation of the presence

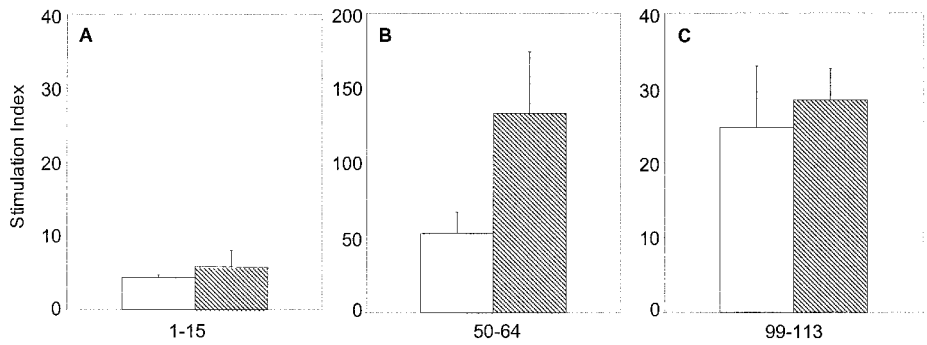

FIGURE 7. Response to defined peptides of ML-M of WT C3H (□) and LysMcre C3H (▨) mice after peptide challenge. Mice (n = 4–6/group) were immunized at the base of tail with a peptide of ML-M in CFA: p1–15 (*A*), p50–64 (*B*), or p99–113 (*C*). Nine days later, the draining LNC of these mice were tested in vitro using the immunizing peptide for recall of T cell response. The results of four to six mice (mean SI units ± SEM) are shown. The PPD response corresponding to these peptides ranged from 24–160.

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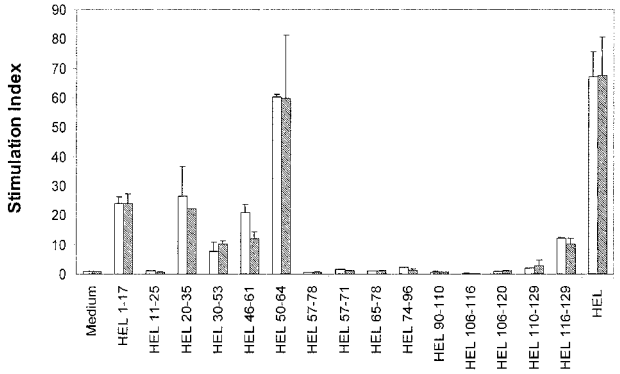

FIGURE 8. Response to HEL of WT C3H (ML-$M^{+/+}$; □) and LysMcre C3H (ML-$M^{-/-}$; ▨) mice immunized with a related homologue of ML-M, HEL. Mice (n = 4/group) were immunized at the base of the tail with HEL/CFA, and on the ninth day the response of LNC was recalled in vitro with HEL or peptides covering the entire length of HEL. The results are shown as mean SI units ± SEM. The response to PPD had a SI value of 62 ± 7 in WT mice and 54 ± 13 in LysMcre mice.

of the T cell repertoire to ML-M and its dominant epitope by experimentally tolerizing these T cells in LysMcre mice; and 4) determination of the impact of epitope-specific tolerance to ML-M on the T cell response to various dominant epitopes within the homologous HEL; in this regard, the rationale for testing the response of LysMcre mice to HEL is significantly different from the use of HEL (or OVA) as a foreign protein in $MBP^{-/-}$ or other Ag-deficient mice (22). In one of the above studies (22), response to homologous gpMBP in $MBP^{-/-}$ mice was tested at the level of whole protein, but not at the epitope level.

LysMcre mice neonatally tolerized with p105–119 failed to raise T cell responses to either p105–119 or ML-M, and thus behaved like WT mice. The lack of response to the two subdominant determinants of ML-M (contained within peptides 95–109 and 110–125) upon adult challenge with ML-M of mice neonatally tolerized with p105–119 ruled out the probability of competition of T cells against different epitopes for interaction with the same APC. Moreover, the absence of cross-reactivity among any of these three peptides (data not shown) excluded cross-tolerance as the reason for the lack of response to these epitopes. The above results suggest that the constraint in revealing these two subdominant epitopes from native ML-M is at the level of Ag processing and not at the level of the T cell repertoire.

Our observation of restimulation (in vitro) of p105–119-primed (in vivo) T cells by WT (ML-$M^{+/+}$) APCs (macrophages) without addition of exogenous Ag (ML-M), but not by LysMcre (ML-$M^{-/-}$) APCs demonstrates that a determinant activating 105–119-specific T cells is spontaneously generated from endogenous ML-M within the macrophages of WT C3H mice. As expected, under the same experimental conditions, the T cells primed by p1–15, which represents a cryptic epitope of ML-M, failed to be restimulated by APC from either WT or LysMcre mice. Constitutive presentation of epitopes from endogenous self Ags has also been reported for other Ags, such as hemoglobin (49) and β_2-microglobulin (50).

There has been a supposition by one group that there is a difference between T cells responding to peptide determinants and those responsive to the same determinant on the native Ag due to many permissible, supposedly conformationally unique, forms of the peptide (51). However, the crucial question in considering the actualities of responsiveness to self is whether in WT mice, T cells directed to the self-dominant determinant(s) are all rendered tolerant or whether clones remain that are only inducible by peptide immunization. Such clones directed against a conformationally unique form of the peptide should be demonstrable by peptide immunization with dominant determinants. However, in the case of ML-M, there are no peptide-inducible T cells remaining to either the dominant determinant 105–119 or the subdominant determinants 95–109 and 110–125 of ML-M in WT C3H mice. This is also true in HEL-tolerant mice of three different haplotypes with respect to the dominant determinants in each case (52) (H. Deng and E. E. Sercarz, unpublished observation).

We observed that WT mice, but not LysMcre mice, are tolerant to self lysozyme. It has been reported that ML-M is expressed in the thymus (17). However, the precise cell type playing a predominant role in thymic selection of the anti-ML-M T cell repertoire remains to be determined. ML-M is synthesized and secreted by cells of the myeloid lineage, including macrophages, granulocytes, and a subgroup of DC closely related to monocyte/macrophage lineage (17, 38). In this regard, ML-M is both an endogenous Ag for cells of the myeloid lineage and a circulating exogenous Ag for these cells as well as for other APC. In the case of another circulating self protein, C5, it has been reported that DC were much more efficient APC than macrophages (10- to 50-fold) and B cells (100- to 500-fold) (20). Similarly, splenic and thymic DC were found to be the most efficient APC, followed by macrophages, large B cells, and small B cells, for the presentation of endogenous naturally processed self epitopes of β_2-microglobulin (50). In another study it was reported that peptides from cytosolic proteins were much more efficiently presented by splenic and thymic DC compared with cortical epithelial cells or resting B cells (53). Also, it has been suggested that DC can efficiently cross-present Ags synthesized and secreted by thymic epithelial cells (45, 54).

Interestingly, ML-M determinants within peptides 1–15, 50–64, and 99–113 that bound well to MHC and were immunogenic in WT mice also induced a potent T cell response in LysMcre mice. These peptides represent cryptic determinants within ML-M. (Of these three ML-M peptides, p1–15 has three amino acid residue differences, whereas the remaining two peptides have only one amino acid residue difference each compared with the corresponding region within the homologous mouse lysozyme-P (ML-P), a 2–4% minor variant of ML-M (17, 55).) These results suggest that T cells against cryptic epitopes of ML-M escaped tolerance induction, and that ML-M is not essential for selecting the T cell repertoire specific for these self-determinants. Although the expression of ML-P is restricted intracellularly within the specialized Paneth cells of the gut (17, 55), we do not know whether ML-P could modulate the selection of ML-M-directed T cells to some extent. We (11, 18, 29, 52, 56) and other investigators (31, 57–60) have previously shown that T cells against cryptic/subdominant epitopes escape tolerance induction. However, only this study has directly addressed and examined the requirement of a particular self Ag for selection of the T cell repertoire against cryptic epitopes of the same Ag. The functional significance of the T cells against cryptic epitopes has been validated by the findings that the display of the previously cryptic or newly emerging epitopes plays a role in the induction/propagation of autoimmunity (29, 31, 32, 61) as well as in graft rejection (33). However, previously nonimmunogenic self epitopes could also be revealed to the immune system in an immunogenic form involving post-translational modification of self epitopes (62). Moreover, other mechanisms besides crypticity have also been invoked in permitting potentially autoreactive T cells to escape thymic negative selection (63–66).

We observed that the absence of ML-M in LysMcre mice did not make any significant difference in the responsiveness of these mice to HEL or to its dominant determinants. Overall, ML-M and

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HEL have 57% amino acid homology, with 36–81% homology between the dominant HEL epitopes tested and the corresponding ML-M epitopes. Thus, a hole in the T cell repertoire caused by ML-M epitopes did not create a parallel hole in the T cell repertoire for HEL. However, at present, the precise TCR Vβ specificities and relative avidities for MHC-peptide complexes of the T cells recruited in response to a particular epitope of HEL (or to a cryptic epitope of ML-M described above) in WT vs LysMcre mice are not defined. In addition, it is not known whether the T cell response to HEL or ML-M in different substrains of C3H (H-2^k) genetic background might be influenced in any significant manner by the presence or the absence of certain components of innate immunity, e.g., the TLR4 (67). The examination of this issue (as well as of the role of CD4$^+$CD25$^+$ T cells in tolerance against ML-M) is currently in progress in our laboratory.

The hierarchy of determinants on a self Ag is established by the rules of processing and presentation, rather than strictly by affinity criteria. Thus, dominant determinants are those that are the most readily accessible to the appropriate MHC groove and have a reasonable affinity for it. These well-expressed determinants will be the ones that induce tolerance and therefore skew the repertoire toward lower avidities by the removal of the highest avidity members. Clearly, in the total absence of a determinant as in a knockout animal or when a large portion of an Ag has been genetically removed, there will be no negative influences on clonal expansion, whereas cross-reactive, positively selecting determinants will probably continue to be a stimulatory influence on homeostatic proliferation. In this report we have shown that the T cell repertoire directed against dominant determinants of self lysozyme in the ML-M knockout mouse, which is usually tolerized in its normal counterpart, fits the criteria we had originally established in considering the self-Ag-specific repertoire and its shaping by the hierarchy of determinants on ML-M (37).

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