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Transport Protein Genes in the Murine MHC: Possible Implications for Antigen Processing

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T lymphocyte activation requires recognition by the T cell of peptide fragments of foreign antigen bound to a self major histocompatibility complex (MHC) molecule. Genetic evidence suggests that part of the class II region of the MHC influences the expression, in trans, of MHC class I antigens on the cell surface, by regulating the availability of peptides that bind to and stabilize the class I molecule. Two closely related genes in this region, *HAM1* and *HAM2*, were cloned and had sequence similarities to a superfamily of genes involved in the ATP-dependent transport of a variety of substrates across cell membranes. Thus, these MHC-linked transport protein genes may be involved in transporting antigen, or peptide fragments thereof, from the cytoplasm into a membrane-bounded compartment containing newly synthesized MHC molecules.

THE LIGAND RECOGNIZED BY THE antigen-specific T cell receptor consists of a peptide fragment of foreign antigen bound to a molecule encoded in the MHC. Peptides bound by MHC class I molecules are recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs), whereas peptides bound to MHC class II molecules are recognized by $CD4^+$ (primarily helper) T cells. Native antigens added exogenously to cells (which are taken up by endocytosis into membrane-bounded vesicles) preferentially yield peptide–class II complexes, whereas endogenously synthesized antigens, or antigens introduced directly into the cytoplasm of living cells, preferentially yield peptide–class I complexes (*1*). However, this distinction is not absolute (*2*). The intracellular pathways taken by antigens destined for processing and presentation to T cells, the exact molecular nature of the physiologic antigenic peptides bound by MHC molecules, and the mechanism of and subcellular compartment for the production of peptides remain unknown.

A mutated murine cell line designated RMA-S, originally selected for loss of class I surface expression (*3*), is defective in the assembly of peptide–class I complexes (*4*). Thus, virally infected RMA-S cells are resistant to lysis by specific CTLs. These cells produce normal class I and β_2-microglobulin mRNA and polypeptides, which, in the absence of bound peptide fragments, fail to associate in a stable manner and are rapidly lost from the cell surface at 37°C. Exogenously added antigenic peptides bind to and stabilize these "empty" class I molecules, thereby rescuing class I surface expression and rendering the cells sensitive to lysis by CTLs (*4*). Although the genetic defect in these cells has not been mapped, the defect in several mutant human cell lines with a similar phenotype (*5*) maps to the class II (*HLA-D*) region of the human MHC. The class II region of the rat MHC also contains a polymorphic, trans-acting gene (called *cim*, for class I modifier)(*6*). Mapping data in the rat system, in which all known MHC genes are colinear with the murine MHC, suggests that the murine equivalent of the *cim* gene maps between the *H-2K* and *I-*A_α genes, most likely between $A_{\beta 3}$ and $A_{\beta 2}$ (*7*). This location is consistent with the location of the deletions in the class I–defective human cell lines. The molecular and genetic evidence thus suggests that the class II region of the MHC contains a gene or genes involved in antigen processing and presentation to class I–restricted T cells. It is possible that the defective gene in RMA-S and the human class I mutants may be identical to the *cim* gene.

The $A_{\beta 3}$ to $A_{\beta 2}$ region of the murine MHC contains at least seven previously undetected genes (*8*). Three of these genes are

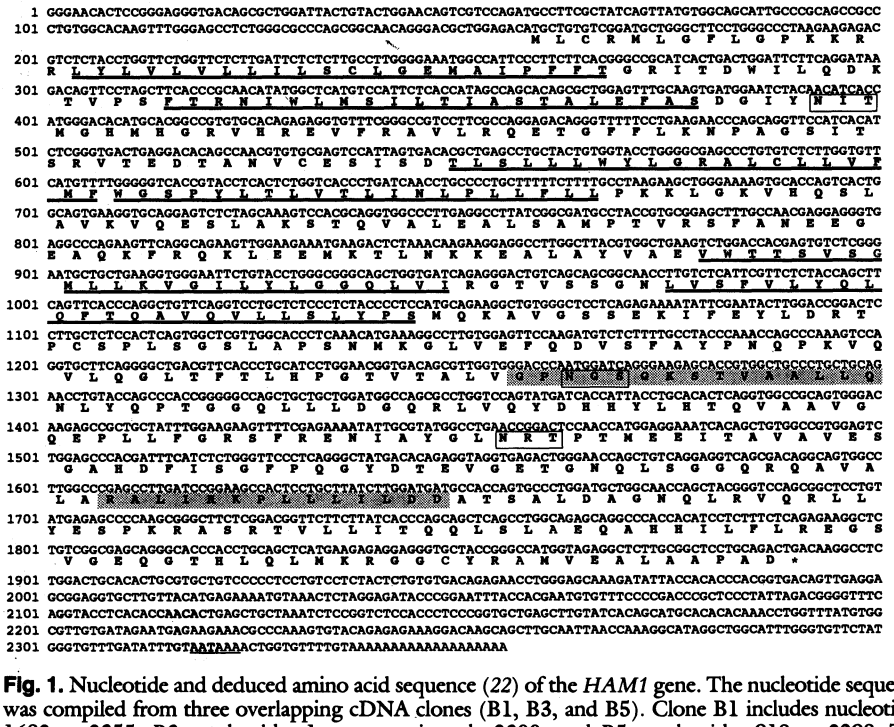

Fig. 1. Nucleotide and deduced amino acid sequence (*22*) of the *HAM1* gene. The nucleotide sequence was compiled from three overlapping cDNA clones (B1, B3, and B5). Clone B1 includes nucleotides 1682 to 2355; B3, nucleotides 1 to approximately 2300; and B5, nucleotides 918 to 2298. The nucleotide sequence of overlapping portions of the three clones that were sequenced are identical except for the G residues at positions 2239 and 2241 in the 3′ untranslated region, which are both T residues in B5. The assignment of these nucleotides as G was confirmed by sequencing cloned BALB/c genomic DNA. In the nucleotide sequence, the polyadenylation signal sequence is underlined and the stop codon identified with an asterisk. In the protein sequence, putative membrane-spanning regions are underlined, potential N-linked glycosylation sites are boxed, and the two regions putatively involved in ATP binding are shaded. Numbering on the left refers to nucleotide positions, and numbering on the right refers to amino acid positions. The GenBank accession number for the nucleotide sequence is M55637.

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highly divergent class II genes (*9*). Characterization of two of the remaining genes suggested a potential mechanism for their involvement in the generation of peptide–class I MHC complexes.

The gene designated *HAM1* (a putative *H*istocompatibility *A*ntigen *M*odifier) spans approximately 10 kb of DNA and lies 30 to 40 kb upstream of $A_{\beta 2}$ (*8*). Northern (RNA) blots probed with genomic fragments of this gene recognized an RNA of approximately 2.6 kb. We isolated cDNA clones corresponding to this mRNA from two independent WEHI-3 (myelomonocytic cell line, H-2^d) cDNA libraries with probe 5.9B, a 4.5-kb Eco RI fragment of genomic DNA containing the 3′ portion of the gene (*8*). The 2355-bp cDNA sequence terminates in a stretch of 19 adenosine residues separated by 13 bp from a consensus AATAAA polyadenylation signal (Fig. 1). A single long open reading frame ends with a TGA termination codon at position 1889. Although no in-frame stop codons appear 5′ of the indicated methionine codon at position 158, the size of this cDNA corresponds closely to the size of the mRNA estimated from Northern blots, given an average of approximately 200 residues in the poly(A)-tail. Therefore, the sequence shown is probably full length, or very nearly so. However, mapping of the transcriptional start site is still in progress, and homologous rat cDNA clones are significantly (about 300 bp) longer at the 5′ end (*10*). With this caveat, the predicted HAM1 protein would contain 577–amino acid residues and have an isoelectric point of 9.2 and a molecular mass of 63,450 daltons. Although this protein contains three potential sites for N-linked glycosylation, all three are predicted to reside in the cytoplasm (on the basis of similarities to known proteins, below) and hence are probably not utilized.

A search of the National Biomedical Research Foundation (NBRF) and Swiss protein databases with the University of Wisconsin Genetics Computer Group sequence analysis programs (*11*) for other homologous proteins reveals similarity of the HAM1 protein to the human, mouse, and hamster multi-drug resistance (mdr) proteins (P-glycoproteins). There are three closely related (71 to 83% amino acid identity) *mdr* genes in the mouse and hamster genomes, and two in humans (*12*). The P-glycoproteins each consist of two similar halves, with each half containing approximately 640 amino acids, which presumably arose by gene duplication (*13*). The predicted HAM1 protein is approximately half the size of P-glycoprotein and shares identities over its entire length with both halves of the murine mdr1 protein. Comparison of the deduced amino acid sequence of *HAM1* with the two halves of the murine mdr1 protein indicated that it is nearly as related to either half (31 to 33% amino acid identity) as the two halves of mdr1 are to one another (39% identity). Hydropathy plots for HAM1 and the COOH-terminal half of mdr1 are virtually superimposable (Fig. 2, A

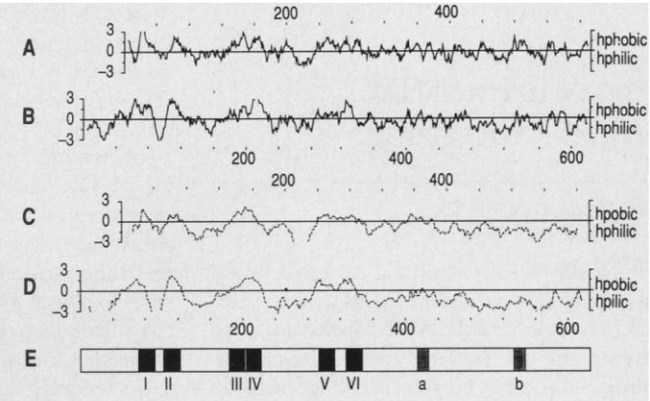

Fig. 2. Comparison of predicted secondary structures of the murine *mdr1* and *HAM1* gene products. Hydropathy plots of HAM1 (**A**) and mdr1 (**B**) were generated by the method of Kyte and Doolittle (*23*). Membrane-spanning regions of HAM1 (**C**) and mdr1 (**D** are predicted by the method of Engelman, Steitz, and Goldman (*24*). (**E**) A schematic showing the relative locations of predicted transmembrane helices (black boxes, I to VI) and the two ATP binding domains (shaded boxes, a and b).

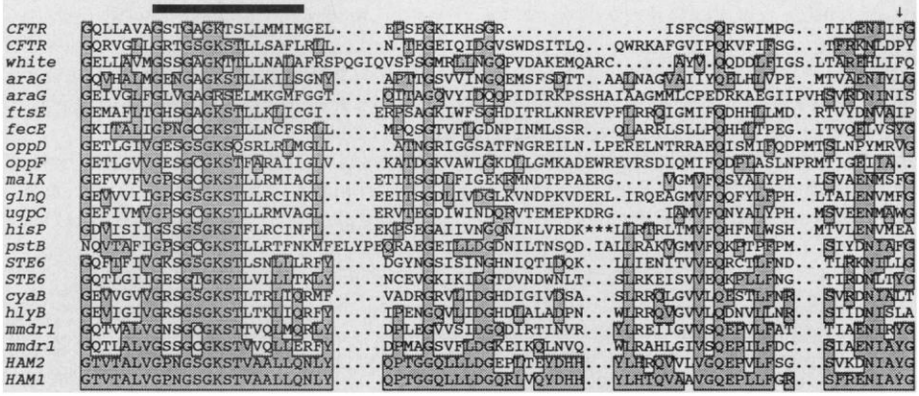

Fig. 3. Comparison of predicted protein sequences of HAM1 and HAM2 with members of the superfamily of related transport proteins. Amino acid residues identical to the residue found in HAM1 are shown in shaded boxes. Gaps introduced into the sequences to facilitate optimal alignment are indicated as dots. The three stars in the *hisP* sequence represent a 13–amino acid deletion introduced for the same purpose. Dark bars at the top of the sequences indicate the positions of the two nucleotide binding consensus sequences, and the arrow indicates the position of the phenylalanine residue in the NH_2-terminal half of the CFTR sequence that is deleted in the majority of cystic fibrosis patients. Numbers in parentheses indicate the percent amino acid identity of each sequence to the HAM1 sequence over the interval shown (amino acids 360–431 and 471–503 of HAM1). For family members that contain homologous halves, the two halves are shown separately, the NH_2-terminal half on the upper, and the COOH-terminal half on the lower line. The proteins shown, their origin, and substrates known or presumed to be transported (*25*) are *CFTR*, human cystic fibrosis transmembrane regulator, chloride ions, or regulators of chloride channels; *white*, *Drosophila melanogaster*, eye pigment; *araG*, *E. coli*, L-arabinose; *ftsE*, *E. coli*, unknown; *fecE*, *E. coli*., iron(III) dicitrate; *oppD* and *oppF*, *Salmonella typhimurium*, oligopeptides; *malK*, *E. coli*, maltose; *glnQ*, *E. coli*, glutamine; *ugpC*, *E. coli*, *sn*-glycerol-3-phosphate and glycerophosphoryl diesters; *hisP*, *S. typhimurium*, histidine, lysine, and arginine; *pstB*, *E. coli*, phosphate; *STE6*, *Saccharomyces cerevisiae*, a-factor mating pheromone; *cyaB*, *B. pertussis*, adenylate cyclase toxin; *hlyB*, *E. coli*, hemolysin; and *mmdr1*, mouse multidrug resistance (P-glycoprotein) gene, cytotoxic drugs. Other superfamily members and their substrates that are not shown include the *Drosophila* brown protein, pigment precursors; *E. coli btuD*, vitamin B12; *E. coli chlD*, molybdate; *Agrobacterium tumefaciens chvA*, β-1,2-glucan; *Rhizobium meliloti ndvA*, β-(1→2)glucan; *Rhizobium leguminosarum nodI*, unknown; *E. coli proV*, glycine betaine and L-proline; and *E. coli rbsA*, ribose (*26*).

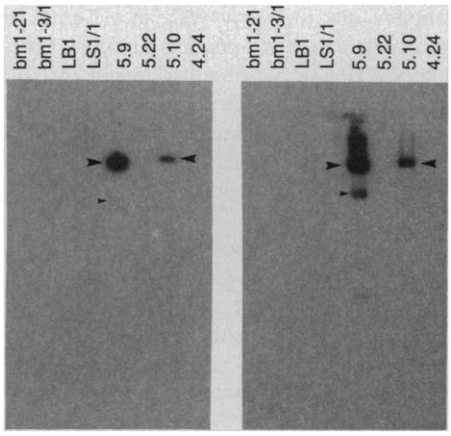

Fig. 4. Southern blot analysis of Eco RI digested cloned mouse genomic DNA probed with 5.9B. Overlapping cosmids LB1, LS1/1, bm1-21, and bm1-3/1 span most of the region centromeric to $A_{\beta 3}$, including *H-2K* and extending to about 70 kb centromeric to *H-2K* (*27*). Cosmids 4.24, 5.22, 5.10, and 5.9 span most of the region between $A_{\beta 3}$ and $A_{\beta 2}$ (*28*). The blot on the left was washed at 55°C in 0.1% SDS and 0.1× SSC, and the blot on the right was washed at 55°C in 0.1% SDS and 1× SSC. Large arrowheads indicate the 4.5-kb fragment (5.9B) containing the 3′ end of the *HAM1* gene, which is present in both 5.9 and the overlapping cosmid 5.10 (*8*). Small arrowheads indicate the 3.3-kb fragment containing the 3′ end of the *HAM2* gene (*8*). The faint bands in the lower portion of the gels are most likely the result of low-level contamination of the cosmid preps with rearranged or partially deleted cosmid DNA (*29*).

and B). Six potential α-helical protein segments are predicted to span lipid bilayers in approximately equivalent locations in both proteins (Fig. 2, C and D). Thus, HAM1 is similar to P-glycoprotein in both primary amino acid sequence and predicted secondary structure.

The *mdr1* gene belongs to a superfamily of genes found both in prokaryotes and eukaryotes that are implicated in the ATP (adenosine triphosphate)-dependent transport of various substrates across cell membranes (*14*). The most highly conserved portions of these molecules reside in the cytoplasmic domains, around two short sequence elements believed to be involved in ATP binding (*15*). A comparison of these regions from 16 members of this family with the corresponding portion of HAM1 (Fig. 3) reveals that the majority of variation between HAM1 and this group of sequences is found at positions that are not highly conserved among the other members of this group.

Complementary DNA clones corresponding to a second gene (*HAM2*) in the region between $A_{\beta 3}$ and $A_{\beta 2}$, which maps approximately 15-kb telomeric of the *HAM1* gene (*8*), cross-hybridize to *HAM1*. No other cross-hybridizing sequences are present in a set of cosmids spanning most of the DNA between *H-2K* and $A_{\beta 2}$, even at moderate (Fig. 4) to low stringency. HAM1 and HAM2 share 77% amino acid identity in the region illustrated in Fig. 3, and most of the residues highly conserved among other family members are also conserved in HAM2. In all but three of the 24 positions at which HAM1 and HAM2 differ, the residue found in HAM2 is also found in at least one other member of the group of sequences shown.

The proteins in this superfamily probably function as units consisting of two transmembrane domains (each containing six segments that span the lipid bilayer) and two cytoplasmic domains (each containing a pair of sequences which form a binding site for ATP) (*14*). All known eukaryotic members of this family (the mammalian and protozoan *mdr*, the cystic fibrosis gene, and the yeast *STE-6*) contain all four of these domains within a single polypeptide. The *HAM1* gene is thus the first eukaryotic member of this family that contains only two domains, similar to several of the prokaryotic members, such as the *Escherichia coli* HlyB (*16*) or *Bordetella pertussis* CyaB (*17*) proteins. The *Salmonella* oligopeptide permease (*18*) has four domains that form the active structure, all found on separate polypeptides; all four of the corresponding genes are required for oligopeptide transport. Thus, although the *hlyB* and *cyaB* gene products probably function as homodimers, the products of the *HAM1* and *HAM2* genes may function as distinct homodimers, a single heterodimer, or as two different heterodimers, each paired with as yet undiscovered members of this gene superfamily.

The identity and mechanism of action of the defective gene in cell lines such as RMA-S that have lost the ability to form peptide-MHC complexes is not known. This phenotype could result from an inability to generate antigenic fragments from native antigen, or from defects in putative molecular chaperones (*19*) that might be postulated to be required for proper folding and assembly of the peptide–class I complex. Our data show that the region of the murine MHC implicated in these mutants contains the genes for two closely related members of a superfamily of ATP-dependent transport proteins. Since antigen that is derived from the cytoplasm must cross a lipid bilayer in order to associate with the external portion of MHC class I molecules, the most likely defect in these cells may be an inability to translocate the antigen (or peptide fragments derived from it) from the cytoplasm into the appropriate membrane-bounded compartment where this association normally takes place. Two prokaryotic members of this gene family, *oppD* and *oppF*, function in the transport of oligopeptides in *Salmonella* (*18*).

The bacterial transporters function via an extracellular substrate-binding protein encoded by a linked gene. Perhaps one or more of the as yet uncharacterized genes in this region (*8*) encodes such a peptide binding protein, or this function might be mediated by an MHC-linked member of the heat shock protein HSP70 family (*20*). Some HSP70-related molecules bind peptides and participate in class II–restricted antigen presentation (*21*). If HAM1 and HAM2 serve as transporters of peptides rather than intact antigens, this implies that peptide need not be generated in the same subcellular compartment in which association with MHC molecule takes place, thus removing the requirement for the presence of antigen processing proteases in the latter compartment. Characterization of the HAM1 and HAM2 proteins and identification of the membranes in which they reside may allow definitive identification of the subcellular location where peptide-MHC complexes form.

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Peripheral Clonal Elimination of Functional T Cells

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A major mechanism for generating tolerance in developing T cells is the intrathymic clonal deletion of T cells that have receptors for those self antigens that are presented on hematopoietic cells. The mechanisms of tolerance induction to antigens not expressed in the thymus remain unclear. Tolerance to self antigens can be generated extrathymically through the induction of clonal nonresponsiveness in T cells with self-reactive receptors. A second mechanism of extrathymic tolerance was identified: clonal elimination of mature T cells with self-reactive receptors that had previously displayed functional reactivity.

CLONAL DELETION IS A MAJOR mechanism to achieve T cell tolerance to self antigens (*1–4*) and is thought to occur only in the thymus (*5, 6*). When this mechanism fails, tolerance may also be acquired by intra- and extrathymically induced clonal nonresponsiveness (anergy) (*7, 8*). The mechanism involved in generating T cell tolerance is probably determined by the manner in which self antigens are presented (*7–9*), and perhaps also by the developmental stage at which T cells encounter these antigens (*10*). Self antigens for which these rules have been established include H-Y antigens (*1*), products of the minor lymphocyte stimulatory (*Mls*) loci and staphylococcal enterotoxins (*2*), and largely unknown gene products that are recognized in association with $E_\alpha E_\beta$–class II molecules of the major histocompatibility complex (MHC) (*3*). In the present study, we established an environment in which both the intrathymic clonal deletion and the clonal anergy pathways were blocked and revealed yet another mechanism of self tolerance. As a model system, we used the Mls-1^a superantigen–$E_\alpha E_\beta$-MHC molecule combination, because potentially Mls-1^a–reactive cells that use $V_\beta 6$ as one of their T cell receptor (TCR) chains can be followed by analyzing $V_\beta 6$ expression among T cells (*2*).

Although some of the requirements for tolerance induction in Mls-1^a reactive cells have been addressed (*7, 11, 12*), several issues remain unresolved. Because some Mls-responsive cells are $CD8^+$, it is not clear how these cells depend on the MHC (*13*). Also, the presumed dependence of clonal deletion on $E_\alpha E_\beta$ in Mls responsive cells was shown only indirectly (*12*), in cell-transfer or chimera experiments. It is unclear whether induction of nonresponsiveness to Mls-1^a (*7, 14, 15*) is dependent on $E_\alpha E_\beta$, or $A_\alpha A_\beta$. Induction of in vitro responsiveness to Mls-1^a can be blocked by both $A_\alpha A_\beta$- and $E_\alpha E_\beta$-specific monoclonal antibodies (MAbs) (*16*).

To address whether Mls-1^a tolerance is dependent on $E_\alpha E_\beta$, Mls-1^a expressing mice [DBA/2 or (DBA/2 × C57Bl/6)F_1] were treated from birth with saturating doses of MAb to E_α, and the presence of potentially Mls-1^a reactive cells was analyzed by following the fate of $V_\beta 6^+$ T cells. MAb-mediated blocking of either class I or class II MHC molecules from birth can abrogate development of CD8 or CD4 expressing T lymphocytes, respectively (*17, 18*). Analysis of E_α and A_β expression in the spleen, lymph nodes, and thymus of treated mice confirmed the complete blocking of recognition of expression of E_α, whereas recognition of A_β was unaffected (*17, 19*). The effects of this treatment on development of the T cell repertoire were analyzed with MAbs specific for the variable (V) region of the T cell receptor β chain and flow cytometry. T cells that expressed $V_\beta 6$ were detected in both the thymus and the periphery of anti-E_α treated mice (Fig. 1) albeit at lower percentages than in $E_\alpha E_\beta^-$ mice, probably due to partial deletion through recognition of Mls-1^a in association with $A_\alpha A_\beta$ (*15*), but not in untreated controls. Additionally, this treatment prevented the deletion of other T cells that do not mature in the presence of $E_\alpha E_\beta$ [that is, $V_\beta 11$ and $V_\beta 5$; (*3, 20*)]. As a control, mice were also analyzed for expression of $V_\beta 14$, which identifies a TCR not dependent upon $E_\alpha E_\beta$ for selection (*21*). The percentage of T cells expressing $V_\beta 14$ was either unaffected or decreased (Fig. 1), most likely reflecting the concomitant increase in T cells with other receptors (V_βs 3, 5, 9, 11, 12, and 16) (*22*) normally deleted in $E_\alpha E_\beta$-expressing mice of the strains used here. The total number of αβ TCR expressing T cells in either the $CD4^+$ or $CD8^+$ subset of T cells was also not affected by the anti-E_α treatment (Fig. 1). Treatment with MAb for the other major class II molecule in DBA/2 mice, $A_\alpha A_\beta$, had no effect (*19*). Thus, $E_\alpha E_\beta$ expression is necessary and sufficient for clonal deletion of $V_\beta 6^+$ T cells.

Subsequent experiments focused on the functional reactivity of the $V_\beta 6^+$ T cells found in anti-E_α–treated mice. The $V_\beta 6^+$ T cells were analyzed both for their response to stimulation through direct crosslinking of their TCRs with MAbs and their response to syngeneic stimulator cells, to test for possible reactivity with self antigens. If, as for deletion, induction of nonresponsiveness

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