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T cell development in mice lacking the CD3-ζ/η gene

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The CD3-ζ and CD3-η polypeptides are two of the components of the T cell antigen receptor (TCR) which contribute to its efficient cell surface expression and account for part of its transducing capability. CD3-ζ and CD3-η result from the alternative splicing of a single gene designated CD3-ζ/η. To evaluate the role of these subunits during T cell development, we have produced mice with a disrupted CD3-ζ/η gene. The analysis of thymocyte populations from the CD3-$\zeta/\eta^{-/-}$ homozygous mutant mice revealed that they have a profound reduction in the surface levels of TCR complexes and that the products of the CD3-ζ/η gene appear to be needed for the efficient generation and/or survival of $CD4^+CD8^+$ thymocytes. Despite the almost total absence of mature single positive thymocytes, the lymph nodes from $\zeta/\eta^{-/-}$ mice were found to contain unusual $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive cells which were $CD3^-$. In contrast to the situation observed in the thymus, the thymus-independent gut intraepithelial lymphocytes present in $\zeta/\eta^{-/-}$ mice do express TCR complexes on their surface and these are associated with FcεRI γ homodimers. These results establish an essential role for the CD3-ζ/η gene products during intrathymic T cell differentiation and further emphasize the difference between conventional T cells and thymus-independent gut intraepithelial lymphocytes.

Key words: gene inactivation/gut intraepithelial lymphocytes/T cell development/TCR–CD3 complex/T lymphocyte

Introduction

The T cell antigen receptor (TCR) complex consists of a clonally variable, antigen binding αβ heterodimer that is non-covalently associated with a group of invariant polypeptides designated CD3-γ, CD3-δ, CD3-ε, CD3-ζ and CD3-η (reviewed by Samelson and Klausner, 1992). All these subunits have to be assembled in a coordinated fashion in the endoplasmic reticulum for efficient transport to the cell surface. The cytoplasmic segments of the various CD3 chains are responsible for coupling the antigen recognition unit to intracellular signalling pathways. Their signal transduction capability has been attributed to the presence of a recurrent motif of ~20 amino acids (reviewed in Malissen and Schmitt-Verhulst, 1993). Each copy of this motif contains a pair of precisely spaced Tyr-X-X-Leu/Ile sequences (where X corresponds to variable residues) and is hereafter referred to as the $(YXXL/I)_2$ motif (Wegener *et al.*, 1992). This motif is triplicated in CD3-ζ and is present as a single copy in the CD3-γ, -δ and -ε chains. As a result of antigen recognition, the two tyrosines found in each motif are phosphorylated and likely to function as bidentate docking sites for the paired SH2 domains present in some lymphocyte-specific tyrosine kinases (e.g. ZAP-70, Chan *et al.*, 1992). Upon recruitment to the inner face of the plasma membrane, these tyrosine kinases appear to be responsible for the initiation of the signalling cascade (reviewed in Weiss, 1993).

The analysis of TCR devoid of functional ζ subunit has led to the view that the TCR complex is composed of at least two parallel signal transducing modules made of the γδε and ζη chains, respectively. Although direct evidence is still lacking for CD3-δ and CD3-γ, it is likely that each of the CD3 polypeptides can individually act as an autonomous transducer once expressed in the context of a whole TCR complex. This modular architecture permits the occurrence of multiple TCR isoforms made of distinct polypeptide combinations. For instance, the γ chain of the high affinity IgE receptor (FcεRI; reviewed in Ravetch and Kinet, 1991) shares significant structural homology with CD3-ζ and CD3-η and could be incorporated in TCR complexes in lieu of CD3-ζ and CD3-η (Orloff *et al.*, 1990; Rodewald *et al.*, 1991; Koyasu *et al.*, 1992; Mizoguchi *et al.*, 1992). Therefore, several TCR isoforms (e.g. $(\alpha\beta)(\delta\epsilon)(\gamma\epsilon)(\zeta)_2$, $(\alpha\beta)(\delta\epsilon)(\gamma\epsilon)(\zeta\eta)$, $(\alpha\beta)(\delta\epsilon)(\gamma\epsilon)(Fc\epsilon RI\gamma)_2$, etc.) may coexist and be responsible for coupling antigen recognition to distinct signalling pathways (Bauer *et al.*, 1991; Kappes and Tonegawa, 1991). An additional level of complexity results from the fact that unique combinations of TCR/CD3 polypeptides appear to be expressed with a temporally restricted pattern. For instance, in contrast to mature T cells, pre-T cell lines can assemble and express on their surface TCR–CD3 complexes devoid of TCR α chain and deprived of or loosely associated with CD3-ζ subunits (Punt *et al.*, 1991; Groettrup *et al.*, 1992; Groettrup and von Boehmer, 1993).

Progenitors entering the thymus carry their TCR loci in germline configuration and may develop along the γδ or αβ T cell lineages. Upon committment to the αβ lineage, immature $CD4^-CD8^-$ ('double negative') thymocytes differentiate into $CD4^+CD8^+$ ('double positive') cells, a small percentage of which mature into either $CD4^+CD8^-$ or $CD4^-CD8^+$ ('mature single positive') cells that correspond to the end stage of the intrathymic differentiation

pathway (reviewed in Shortman, 1992). Rearrangement at the TCR β locus occurs at the double negative stage and precedes rearrangement at the TCR α locus. Maturation from the double negative compartment as well as expansion of the resulting early $CD4^+CD8^+$ cells appear to require TCR β chain expression and to be totally independent of TCR α chain expression (Kishi *et al.*, 1991; Mombaerts *et al.*, 1992a; Philpott *et al.* 1992; Mallick *et al.*, 1993; Shinkai *et al.*, 1993; Shores *et al.*, 1993). It is not yet clear whether the critical role played by the TCR β chain relies on its intracellular display or requires its surface expression in association with some CD3 subunits (Levelt *et al.*, 1993; Ossendorp *et al.*, 1992; Groettrup and von Boehmer, 1993). Experiments with transgenic mice carrying productively rearranged TCR genes have indicated that positive selection (i.e. the transition from the double positive to the single positive stage) is determined by the specificity of the clonally variable TCR $\alpha\beta$ dimer and its interplay with a set of surface molecules among which CD4, CD8 and major histocompatibility complex (MHC) molecules play a determining role (reviewed in von Boehmer and Kisielow, 1993).

The above data suggest the existence of developmental control points (Shortman, 1992; Chan *et al.*, 1993; Davis *et al.*, 1993) which ensure that most T cells do not complete their intrathymic differentiation program in the absence of productive TCR gene rearrangements or if they express TCR $\alpha\beta$ combinations with inappropriate specificities (i.e. TCR which are self-reactive, unable to cooperate with the set of coexpresssed self-MHC molecules or displayed with a mismatched CD4/CD8 coreceptor molecule). These 'proofreading' mechanisms probably operate via the triggering of the TCR−CD3 complexes expressed at the surface of developing T cells. By activating intracellular effectors, TCR engagement may modulate gene expression pattern and induce the aforementioned phenotypic shifts (Bendelac and Schwartz, 1991; Turka *et al.*, 1991; Anderson *et al.*, 1992; Borgulya *et al.*, 1992; Nakayama and Loh, 1992; Nakayama *et al.*, 1992; Swat *et al.*, 1992; Takahama

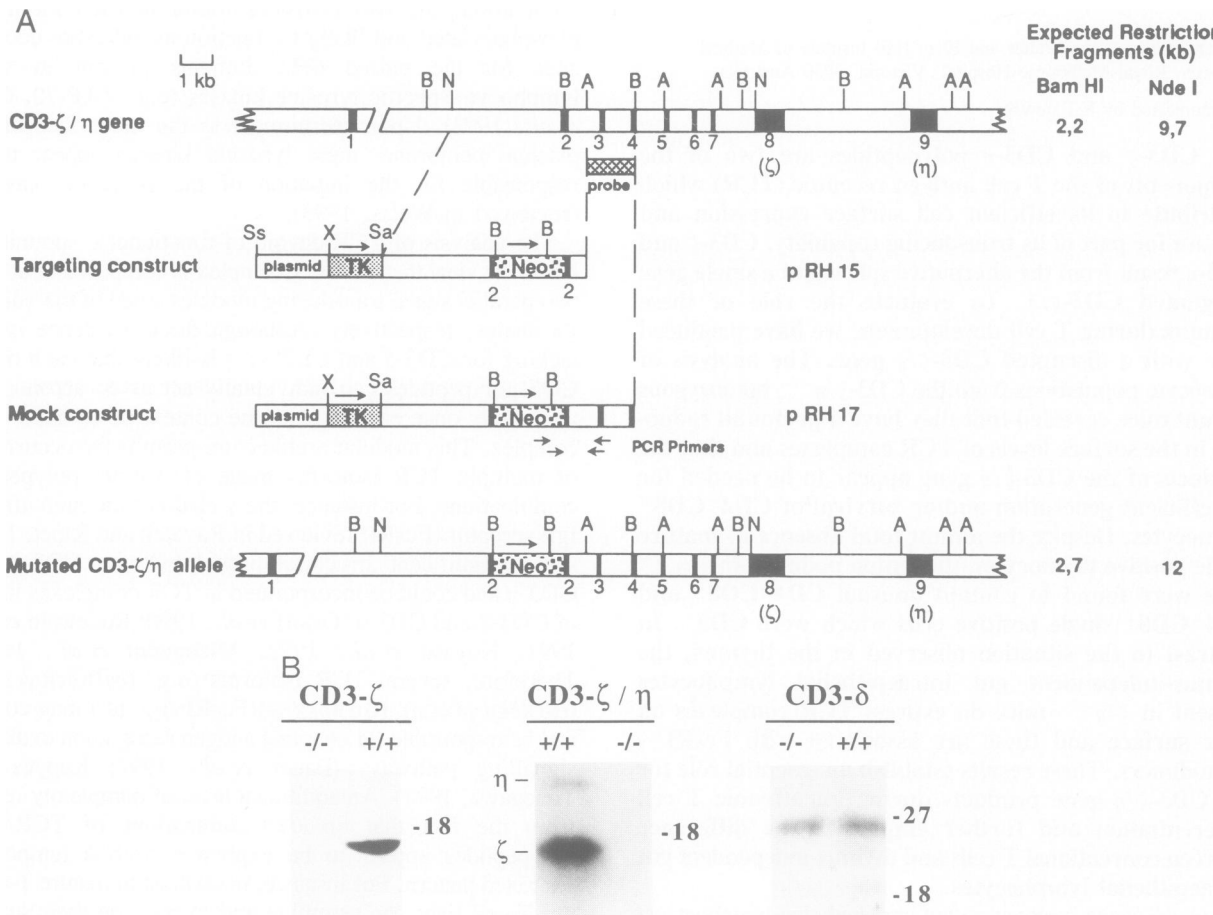

Fig. 1. Disruption of the CD3 ζ/η gene by homologous recombination and analysis of CD3 ζ/η protein expression. (**A**) The first line shows the exon−intron organization of the mouse CD3 ζ/η gene. Exons are depicted by filled boxes and numbered 1 to 9. The size of intron 1 has not yet been determined. The targeting construct (pRH15) used to disrupt the ζ/η gene corresponds to a 4.3 kb *Nde*I−*Acc*I fragment into which a pgk-neo cassette (neo) has been inserted at a *Bam*HI site located within exon 2. The resulting construct was abutted to a TK expression cassette (TK) and electroporated into ES cells (see Materials and methods). The structure of the disrupted gene is indicated on the bottom line. Also shown are the structure of a mock construct (pRH17) used as a control template during PCR analysis, as well as the location of two pairs of PCR primers used to screen for homologous recombinants (arrows below the mock construct diagram). Homologous recombinants were confirmed by restriction enzyme digestion and Southern blotting using a 1.4 kb *Acc*I−*Bam*HI genomic fragment as a probe. This probe, the position of which is shown below the CD3 ζ/η gene (probe), hybridizes on *Bam*HI-digested DNA to a 2.2 kb wild-type fragment and to a 2.7 kb recombinant fragment. Horizontal arrows above the neo and TK genes indicate 5′ to 3′ orientation. The position of relevant restriction sites are indicated by vertical lines: A, *Acc*I; B, *Bam*HI; N, *Nde*I; Sa, *Sal*I; Ss, *Sst*II; and X, *Xho*I. (**B**) Western blotting of $\zeta/\eta^{+/+}$ and $\zeta/\eta^{-/-}$ thymocytes. Whole thymocyte lysates were run under reducing conditions on an 8% SDS−polycacrylamide gel, blotted and probed with either a rabbit antiserum that detects both the CD3-ζ and CD3-η polypeptides (panel CD3 ζ/η), or with an anti-CD3-ζ monoclonal antibody (H146-968, panel CD3 ζ). A control blot was run in parallel and probed with an antiserum against mouse CD3-δ. The positions of two molecular weight standards are indicated (in kDa).

and Singer, 1992; Takahama *et al.*, 1992; Chan *et al.*, 1993; Davis *et al.*, 1993; Levelt *et al.*, 1993; Penninger *et al.*, 1993). As a first step toward the understanding of the role played by the CD3 subunits during T cell development, we have generated mice that do not express the products of the CD3-ζ/η gene and characterized their T cell compartment.

Results

Generation of CD3-ζ/η deficient mice

The CD3-ζ/η gene contains nine exons and gives rise to two alternative splice products designated CD3-ζ and CD3-η (Baniyash *et al.*, 1989; Ohno and Saito, 1990; Clayton *et al.*, 1991). The transcripts corresponding to CD3-ζ include exons 1−8, whereas those corresponding to CD3-η arise when exon 8 is replaced by a downstream η-specific exon (exon 9 in Figure 1A), As a consequence, the ζ and η polypeptides are identical for 122 amino acids and then display distinct carboxy-terminal ends. To produce mice lacking the CD3-ζ and -η polypeptides, the CD3-ζ/η gene was mutated by introducing an expressible neomycin gene into a *Bam*HI site located within the exon coding for the transmembrane segment (exon 2, Figure 1A). This construct should disrupt the reading frame of the gene. Moreover, owing to the fact that the introns flanking exon 2 belong to two different classes (Baniyash *et al.*, 1989), the occurrence of adventitious splicing events connecting exons 1 and 3 should result in the simultaneous loss of the proper translational reading frame. The targeting vector was electroporated into the Bruce 4 embryonic stem cell line (Köntgen *et al.*, 1993). Clones resistant to both neomycin and gancyclovir were screened by polymerase chain reaction (PCR) for homologous recombination events. Two homologous recombinant clones were identified out of 760 tested colonies. Germline transmission of the mutation was obtained with one of the embryonic stem cell clones. Heterozygous offspring mice were derived from the founder and used to produce individuals homozygous for the mutated CD3-ζ/η allele. Homozygous mutant mice appeared healthy and were bred under pathogen-free conditions. To establish that the mutation indeed prevented the expression of the ζ and η polypeptides, thymocyte lysates were analyzed by Western blotting using either a monoclonal antibody directed against CD3-ζ (Punt *et al.*, 1991), or a rabbit antiserum raised against a peptide common to ζ and η (Orloff *et al.*, 1990). Homozygous mutant mice were found to lack both ζ and η polypeptides (Figure 1B).

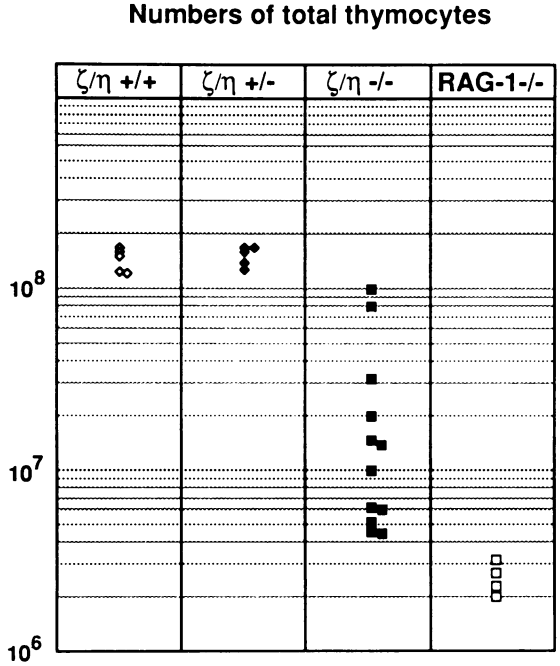

Fig. 2. Numbers of thymocytes found in wild-type littermates ($\zeta/\eta^{+/+}$) and mice heterozygous ($\zeta/\eta^{+/-}$) or homozygous ($\zeta/\eta^{-/-}$) for the disrupted CD3-ζ/η gene. RAG-$1^{-/-}$ mutant mice were also included for comparison. The total numbers of thymocytes found in individual mice were plotted on a logarithmic scale. Mice were between 4 weeks and 3 months old. $\zeta/\eta^{+/+}$: n = 5, average 1.5 × 10^8; $\zeta/\eta^{+/-}$: n = 5, average 1.5 × 10^8; $\zeta/\eta^{-/-}$: n = 12, average 2.4 × 10^7; RAG-$1^{-/-}$, n = 4, average 2.5 × 10^6.

Thymocyte populations in ζ/η-deficient mice

Substantial interindividual variations were unexpectedly observed in the total numbers of thymocytes found in CD3-$\zeta/\eta^{-/-}$ mice. As shown in Figure 2, $\zeta/\eta^{-/-}$ thymuses contained from 2- to 30-fold less cells than those of wild-type or $\zeta/\eta^{+/-}$ heterozygous mice. When analyzed by two-color flow cytometry, $\zeta/\eta^{-/-}$ thymuses were found to contain 13−30% double negative and 60−80% double positive cells (Figure 3). Due to the reduced cellularity of the $\zeta/\eta^{-/-}$ thymuses, this increase in the percentage of double negative cells corresponded to a reduction in the numbers of double positive cells. As shown in Figure 3, there were dramatically fewer single positive cells than in wild-type mice.

Double negative cells can be divided into different subsets based on the differential expression of the interleukin-2 receptor α chain (CD25) and heat stable antigen (HSA). In wild-type mice, the $CD25^+$ population represented only ~2% of thymocytes (Figure 4). Conversely, in mice deficient in at least one of the two recombinase-activating genes (RAG-1 or RAG-2), the majority of the thymocytes were blocked at the $CD25^+$ stage (Figure 4; Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1992). As shown in Figure 4, the $\zeta/\eta^{-/-}$ thymocytes contained approximately similar percentages of $CD25^-$ (46%) and $CD25^{high}$ (34%) cells. Moreover, a streak of cells (20%) expressing intermediate levels of CD25 ($CD25^{intermediate}$) and extending from the position of the $CD25^-$ cells to that of the $CD25^{high}$ cells was reproducibly found in the ζ/η mutant mice (Figure 4). The $CD25^-$ cells were mostly $CD4^+CD8^+$, whereas the $CD25^{high}$ cells were either $CD4^-CD8^-$ (for the majority) or $CD4^{low}CD8^{low}$ (data not shown). Interestingly, the $CD25^{intermediate}$ scored within the $CD4^+CD8^+$ window. However, the levels of CD4/CD8 molecules they expressed were on average slightly lower than those found on the $CD4^+CD8^+CD25^-$ cells. The crescent shape of the dot plot of HSA versus CD25 observed in $\zeta/\eta^{-/-}$ mice suggested the presence of a discrete HSA^{bright} subpopulation (Figure 4). Triple-color analysis confirmed the existence of the latter and further demonstrated that it constitutes 15−17% of the $\zeta/\eta^{-/-}$ thymocytes and presents a $CD4^{low}CD8^{low}$ phenotype (data not shown). A similar $HSA^{bright}CD4^{low}CD8^{low}$ subpopulation, representing <2% of the total thymocytes, was also found in wild-type littermates (data not shown). Taken together, these data suggest that the CD3 ζ/η mutation partially impedes the progression to the double positive stage and/or the coincident expansion of the corresponding 'transitional' thymocytes.

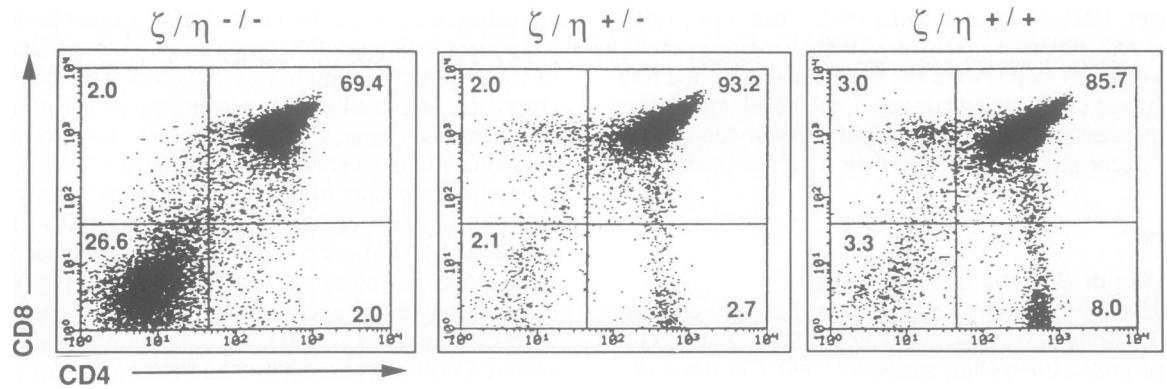

Fig. 3. The CD3-ζ/η mutation decreases the transition of thymocytes from the CD4$^-$CD8$^-$ stage to the CD4$^+$CD8$^+$ stage. Thymocytes from a wild-type littermate ($\zeta/\eta^{+/+}$) or from a mouse heterozygous ($\zeta/\eta^{+/-}$) or homozygous ($\zeta/\eta^{-/-}$) for the disrupted CD3-ζ/η gene were analyzed by two-color flow cytometry for the expression of CD4 versus CD8. The percentage of cells found in each quadrant is indicated. All the $\zeta/\eta^{-/-}$ thymocytes were found to be Thy1$^+$ (data not shown).

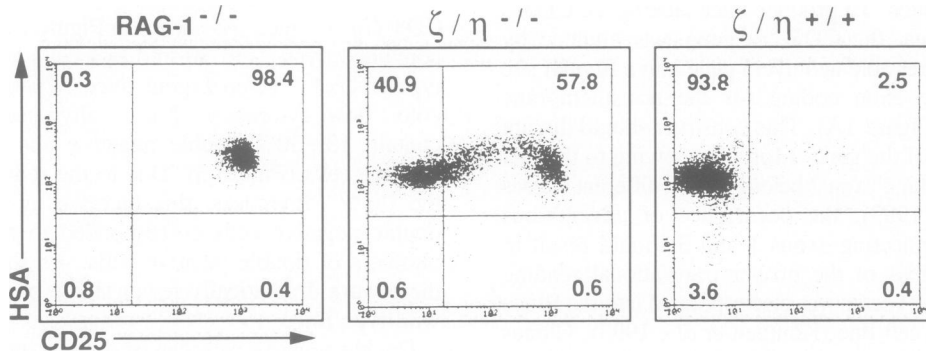

Fig. 4. T cell maturation in CD3-$\zeta/\eta^{-/-}$ mice. Thymocytes from RAG-1$^{-/-}$, CD3-$\zeta/\eta^{-/-}$ or wild-type ($\zeta/\eta^{+/+}$) adult mice were analyzed by two-color flow cytometry for expression of heat-stable antigen (HSA) and IL-2R α chain (CD25). The percentage of cells found in each quadrant is indicated.

Southern blot analysis of $\zeta/\eta^{-/-}$ thymocytes showed that the TCR α- and TCR β-rearrangements were as extensive as those in wild-type or $\zeta/\eta^{+/-}$ heterozygous mice (data not shown). In support of the Southern blot data, full-length TCR α- and TCR β-transcripts were readily detectable in $\zeta/\eta^{-/-}$ mice (Figure 5). Furthermore, when analyzed by Western blot and probed with a rabbit antiserum directed to the TCR Cα domain, thymus lysates from $\zeta/\eta^{-/-}$ mice were found to contain a 46 kDa band which probably corresponded to intracytoplasmic TCR α chains (data not shown). Therefore, the CD3-ζ/η gene products appear unnecessary for the transcription and translation of TCR genes.

To determine whether the ζ/η mutation abolishes TCR expression at the surface of developing T cells, thymocytes from $\zeta/\eta^{+/+}$ and $\zeta/\eta^{-/-}$ mice were stained with a monoclonal antibody directed against the constant region of the TCR β chain. As shown in Figure 6, the ζ/η mutation resulted in an almost complete disappearance of T cells expressing low or high levels of TCR complexes. However, when compared with a negative control histogram obtained after staining thymocytes from a TCR $\beta^{-/-}$ mouse (Mombaerts *et al.*, 1992a), the $\zeta/\eta^{-/-}$ thymocytes appeared to display low levels of staining which are comparable in intensity with those observed on thymocytes from TCR $\alpha^{-/-}$ mice (Mombaerts *et al.*, 1992a). Similar results were obtained with a set of anti-CD3-ϵ monoclonal antibodies (data not shown). In addition, staining with an anti-TCR δ antibody failed to detect TCR $\gamma\delta^+$ cells in $\zeta/\eta^{-/-}$ adult thymuses (data not shown). It is worth noticing that the surface levels of TCR complexes found on the thymocytes of $\zeta/\eta^{+/-}$ heterozygous mice were about half that of wild-type littermates (data not shown). This 2-fold reduction does not seem to have a noticeable effect on intrathymic differentiation (Figure 3). However, it should be noted that some of the $\zeta/\eta^{+/-}$ thymuses (for instance the one shown in Figure 3) contained a lower percentage

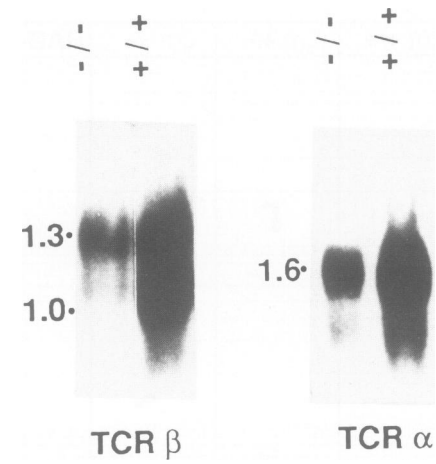

Fig. 5. Northern blot analysis of thymocytes from CD3-$\zeta/\eta^{-/-}$ and CD3-$\zeta/\eta^{+/+}$ littermates. Blots were hybridized with probes corresponding to Cβ or Cα. The relative size of the bands are indicated in the margins. The 1.0 kb TCR β transcripts appear to result from partial DβJβ rearrangements.

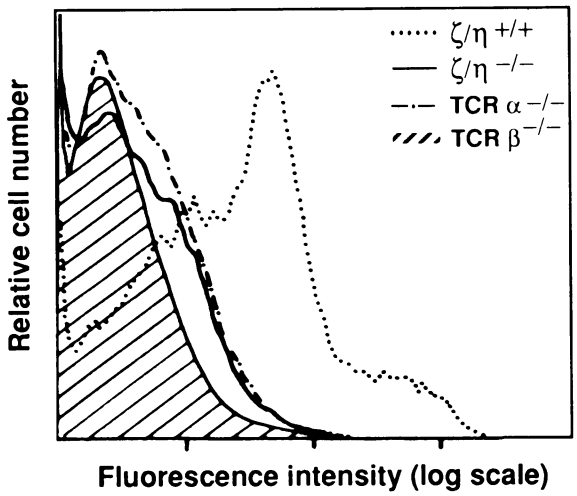

Fig. 6. Minute amounts of TCR β chain on the surface of thymocytes from CD3 $\zeta/\eta^{-/-}$ mice. Thymocytes were isolated from CD3 $\zeta/\eta^{-/-}$, TCR $\alpha^{-/-}$, TCR $\beta^{-/-}$ or wild-type ($\zeta/\eta^{+/+}$) mice and stained with an anti-TCR β (H57-597) antibody. The negative control fluorescence profile (shaded area) corresponds to that obtained after staining TCR $\beta^{-/-}$ thymocytes with H57-597.

of $CD4^+CD8^-$ mature T cells than did their $\zeta/\eta^{+/+}$ littermates.

We next compared the $\zeta/\eta^{-/-}$ thymocytes with those found in mice carrying mutations in the genes coding for some of the TCR components (Mombaerts *et al.*, 1992a,b; Philpott *et al.*, 1992; E.Spanopoulou, unpublished results). Based on the expression of the CD4 and CD8 molecules, as well as on total cell number counts, the CD3-ζ/η mutation appeared to block thymocyte differentiation at an earlier stage than the mutation in TCR α, and did not affect the progression to the double positive stage as much as the TCR β mutation (Figure 7).

T cell subpopulations in lymph nodes from ζ/η-deficient mice

The lymph nodes of $\zeta/\eta^{-/-}$ mice were slightly enlarged and contained reduced percentages of $Thy1^+$ cells (from 7 to 27% of the total number of cells). When analyzed for surface expression of CD4 and CD8, B cell depleted lymph nodes were found to comprise both single positive and double negative cells (Figure 8). The former were $CD3^-HSA^-$ and over 90% expressed the CD44 molecule, a surface marker thought to correlate with an activated cell status. A small fraction (~10%) of the $CD4^-CD8^-$ cells, the developmental origin of which has yet to be determined, was brightly stained with an anti-CD3-ε antibody (2C11) and included both TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ cells (data not shown).

Gut intraepithelial lymphocytes in CD3 $\zeta/\eta^{-/-}$ mice

In normal mice, the $CD3^+$ gut intraepithelial lymphocytes (IEL) can be subdivided according to their site of origin (reviewed in Guy-Grand and Vassalli, 1993). A fraction of them belongs to a thymus-dependent TCR $\alpha\beta^+$ lineage and expresses either the CD4 or CD8 αβ molecules. The remaining $CD3^+$ gut IEL are TCR $\alpha\beta^+$ or TCR $\gamma\delta^+$ and may coexpress CD8 αα homodimers. They appear able to differentiate via an extrathymic pathway and follow rules of repertoire selection which are different from those followed by the thymus-dependent TCR $\alpha\beta^+$ CD8 αβ or $CD4^+$ gut IEL subset.

Gut IEL from $\zeta/\eta^{-/-}$ and control littermates were analyzed by three-color cytofluorometry using various combinations of antibodies specific for the CD4, CD8 α, CD8 β, CD3-ε, TCR αβ or TCR γδ molecules. The numbers of total gut IEL recovered from ζ/η-deficient mice were comparable with those found in normal animals. In contrast to the situation observed in the thymus, $CD3^+$ cells were readily detectable in the IEL population from ζ/η-deficient mice (Figure 9). The percentages of $CD3^+$ cells were always lower than in normal littermates and showed substantial interindividual variations. These $CD3^+$ cells expressed CD8 αα homodimers and consisted of both TCR $\alpha\beta^+$ and TCR $\gamma\delta^+$ cells (Figure 9). When compared with those found in normal littermates, the gut IEL from $\zeta/\eta^{-/-}$ mice expressed consistently lower surface levels of TCR αβ, TCR γδ and CD3-ε chains (Figure 9). As shown in Figure 10, the $CD4^+CD8^-$ and $CD4^-CD8$ $\alpha\beta^+$ subsets were also represented in the gut IEL from ζ/η-deficient mice. However, in contrast to the situation observed in $\zeta/\eta^{+/+}$ littermates, both of these subsets were found to be TCR $\alpha\beta^-$ (Figure 10). Therefore, the majority of the TCR $\alpha\beta^+$ and TCR $\gamma\delta^+$ cells found in the gut IEL from $\zeta/\eta^{-/-}$ mice do not express CD4 or CD8 β and probably belong to the lineage characterized as thymus-independent in normal mice.

To determine whether the TCR−CD3 complexes detected on the gut IEL from ζ/η-deficient mice contained FcεRI γ homodimers, gut IEL were isolated and lysed with digitonin to preserve any weak associations between the TCR/CD3 subunits. TCR complexes were then immunoprecipitated with an anti-CD3-ε antibody, run on non-reducing SDS−polyacrylamide gel, blotted and finally probed with an antiserum specific for the mouse FcεRI γ chain (Orloff *et al.*, 1990). As shown in Figure 11, lane anti-CD3-ε, a band which migrates with the predicted molecular weight of FcεRI γ homodimers (~18 kDa) was found associated with the TCR−CD3 complexes expressed in the gut IEL from ζ/η-deficient mice. Note that this band is absent from immunoprecipitations performed with an isotype-matched, negative control antibody (H146-968: anti-mouse CD3-ζ; Figure 11, lane anti-CD3-ζ). Therefore, the gut IEL from $\zeta/\eta^{-/-}$ mice appear to express TCR complexes associated with FcεRI γ homodimers. Moreover, overexposure of the gel shown in Figure 11 revealed the presence of a faint band of 18 kDa in anti-CD3-ε immunoprecipitates from normal gut IEL (data not shown) which suggested that FcεRI γ homodimers probably coexist with CD3-ζ homodimers on normal gut IEL.

Discussion

This work indicates that the products of the CD3-ζ/η gene are differentially involved in the intrathymic and extrathymic pathways of T cell development. It also suggests that early intrathymic differentiation events can proceed normally in the absence of ζ and η polypeptides. In marked contrast, disruption of the CD3 ζ/η gene appears to impede partially the exit from the double negative compartment and/or the survival and expansion of the resulting double positive cells. The introduction of a TCR β transgene into $RAG^{-/-}$ mice has been shown to result in the complete restoration of the double positive cell population (Mombaerts *et al.*, 1992a;

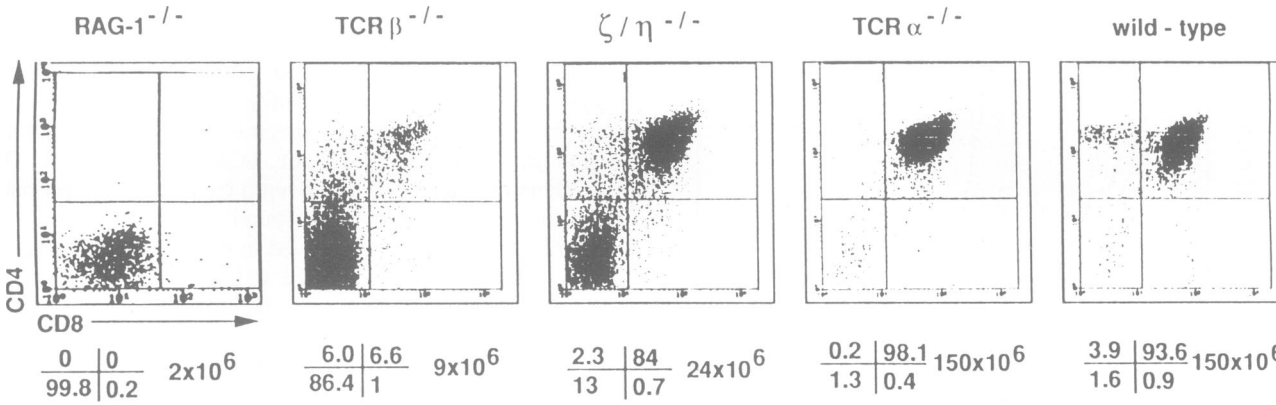

Fig. 7. The CD3-ζ/η mutation blocks $\alpha\beta$ thymocyte development at a later stage than a TCR β mutation but at an earlier stage than a mutation in TCR α. Thymocytes isolated from RAG-$1^{-/-}$, TCR $\beta^{-/-}$, $\zeta/\eta^{-/-}$, TCR $\alpha^{-/-}$ and wild-type mice were analyzed by flow cytometry for the expression of CD4 versus CD8. The percentage of cells found in each quadrant as well as the averaged numbers of thymocytes found in each mouse are indicated under the corresponding dot displays.

Shinkai *et al.*, 1993). The latter expressed weak levels of surface TCR β chains which were found associated with CD3-γ, -δ and -ϵ but not CD3-ζ or -η. On that basis, it has been concluded that the products of the CD3-ζ/η gene are dispensable to the progression to the double positive stage (Shinkai *et al.*, 1993). Implicit in this conclusion was the idea that the α-less TCR complexes detected at the surface of developing T cells may have used their CD3-$\gamma\delta\epsilon$ module to induce the maturation out of the double negative stage (see also Groettrup *et al.*, 1992). Accordingly, CD3-$\zeta/\eta^{-/-}$ thymuses should have displayed a CD4/CD8 phenotype and a cell content similar to those found in both (TCR β^{+}, RAG$^{-/-}$) and TCR $\alpha^{-/-}$ mice. The comparative analysis shown in Figure 7 clearly indicates that this is indeed not the case and further suggests that the mutation in CD3-ζ/η is epistatic to that in TCR α (note that a formal demonstration of this last point will require TCR $\alpha^{-/-}$ × CD3-$\zeta/\eta^{-/-}$ crosses). Interestingly, the disruption of the CD3-ζ/η gene does not completely abrogate the progression to the double positive stage but rather slows it down or limits its extent. This is reflected by a 2- to 4-fold increase in the absolute numbers of cells with a 'transitional' phenotype (i.e. HSA^{bright}, CD4^{low}, CD8^{low}, CD25^{bright} to $^{-}$, Figure 4) and thus contrasts with the stringent block imposed by the TCR β mutation. Note that the double positive cells found in TCR $\beta^{-/-}$ mice (see Figure 7) are absent in both (TCR $\beta^{-/-}$, TCR $\delta^{-/-}$) and (TCR α^{+}, RAG$^{-/-}$) mice and therefore probably belong to the $\gamma\delta$ T cell lineage (Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1993). Taken together, these data suggest that the products of the CD3-ζ/η gene synergize in an as yet undefined way, with the TCR β polypeptides expressed in immature T lymphocytes. In support of this conclusion and in contrast to the data of Shinkai *et al.* (1993), some of the α-less TCR complexes expressed at the surface of pre-T cell lines have been found loosely associated to CD3-ζ (Punt *et al.*, 1991). Finally, it is worth emphasizing that the large variations observed in the size of the $\zeta/\eta^{-/-}$ thymuses may be due to the fact that this mutation affects a developmental phase which is characterized by a high incidence of dividing cells (Shortman *et al.*, 1990).

As previously hypothesized for TCR α-deficient mice (Mombaerts *et al.*, 1992a; Philpott *et al.*, 1992), the failure to express normal levels of TCR at the surface of double positive $\zeta/\eta^{-/-}$ thymocytes probably accounts for the near total absence of mature single positive thymocytes. A similar

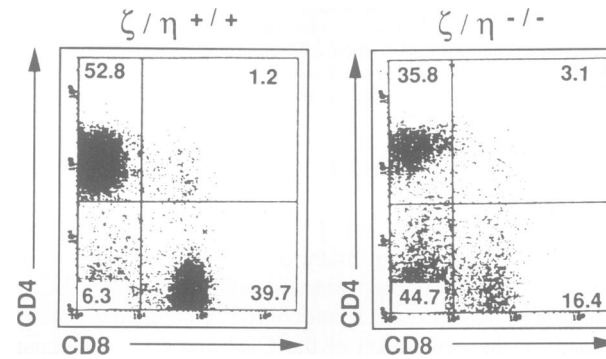

Fig. 8. Lymph node T cells from CD3-$\zeta/\eta^{-/-}$ mice contain CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} subpopulations. Lymph node cells were depleted of B cells using anti-Ig-coated Dynabeads and analyzed by two-color flow cytometry for the expression of CD4 versus CD8. The percentage of cells found in each quadrant is indicated.

lack of mature single positive thymocytes has been documented in mice deficient in both MHC class I and class II molecules (Chan *et al.*, 1993; Grusby *et al.*, 1993). However, in marked contrast to the latter, the ζ/η deficient mice do not show a massive depletion of their peripheral single positive cells. The expression of the CD8 $\alpha\beta$ isoform or CD4 molecule at the surface of the CD3^{-}HSA^{-}CD44^{+} Thy1^{+} peripheral T cells found in $\zeta/\eta^{-/-}$ mice suggests that they have matured via a thymus-dependent pathway (see Rocha *et al.*, 1992; Torres-Nagel *et al.*, 1992). Therefore, it is possible that even the very low levels of TCR detected at the surface of the $\zeta/\eta^{-/-}$ thymocytes may have been sufficient to interact with MHC molecules, trigger the maturation of a few T cells and promote their accumulation over time in the periphery. According to this model, these CD3^{-} single positive peripheral T cells should be absent in CD3-$\zeta/\eta^{-/-}$ × MHC class I and class II$^{-/-}$ crosses. Along this line, it is noteworthy that the minute amount of ζ/η-deficient TCR complexes expressed at the surface of the MA5.8 T cell hybridoma are still capable of triggering weak activation in response to high antigen concentrations (Hermans and Malissen, 1993). Alternatively, the CD3^{-} single positive cells found in the lymph nodes of the ζ/η-deficient mice may have been induced to mature via the Thy1, Ly-6, CD2 or CD44 activation pathways (Seth *et al.*, 1991; Hermans and Malissen, 1993), and may possibly be

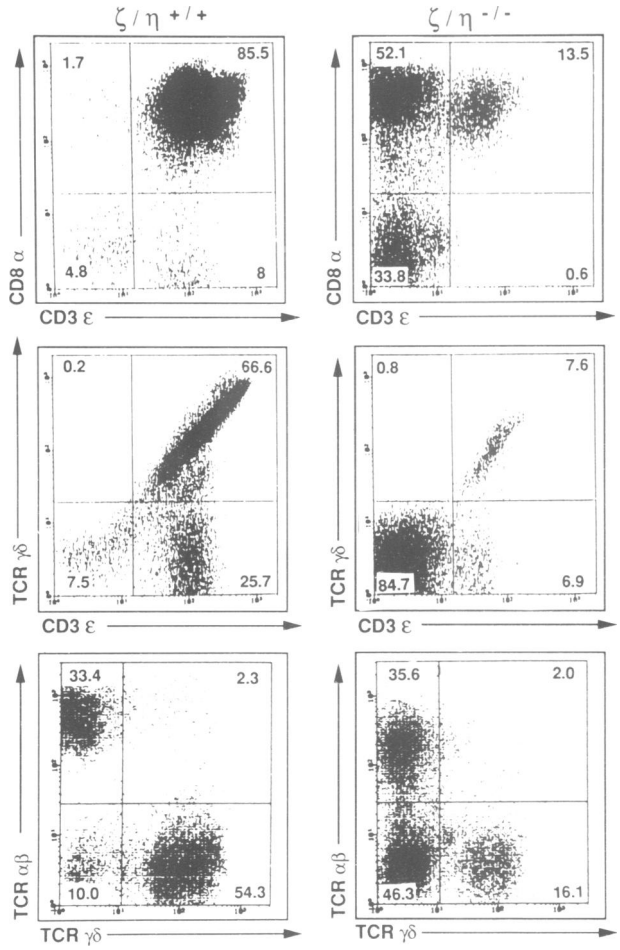

Fig. 9. TCR/CD3$^+$ cells are present in the gut IEL of CD3-ζ/η-deficient mice. IEL from either CD3-$\zeta/\eta^{-/-}$ mice or control littermates ($\zeta/\eta^{+/+}$) were analyzed by two-color flow cytometry for the expression of CD8 α versus CD3-ε (top row), TCR γδ versus CD3-ε (middle row) and TCR αβ versus TCR γδ (bottom row).The percentage of cells found in each quadrant is indicated. The mouse used to generate the top and middle rows contained 14.5% CD3$^+$ cells, whereas that used to generate the bottom row as well as Figure 10 contained 51.7% CD3$^+$ cells.

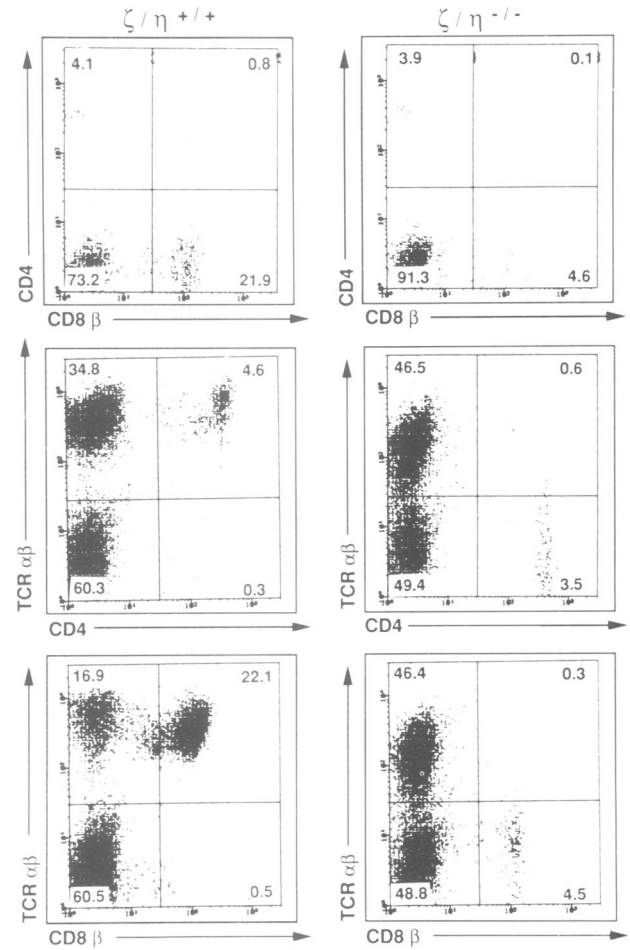

Fig. 10. The few CD4$^+$CD8$^-$ and CD4$^-$CD8 αβ$^+$ gut IEL found in CD3-ζ/η-deficient mice are TCR$^-$. IEL from either a CD3-$\zeta/\eta^{-/-}$ mouse or a control littermate ($\zeta/\eta^{+/+}$) were analyzed by flow cytometry for the expression of CD4 versus CD8 β (top row), TCR αβ versus CD4 (middle row) and TCR αβ versus CD8 β (bottom row). The percentage of cells found in each quadrant is indicated.

related to the Thy1^{low}CD3$^-$CD4$^+$CD8$^-$CD44$^+$ population identified in normal lymph nodes (Kelly and Scollay, 1992).

Our analysis also allowed us to determine whether the FcεRI γ chain is able to substitute for the ζ and η polypeptides and support T cell development. Northern blot analysis performed on thymuses from 1–4 month old $\zeta/\eta^{-/-}$ mice failed to detect any FcεRI γ transcripts (data not shown), and provided an explanation for the fact that adult intrathymic T cell development could not be rescued through the utilization of FcεRI γ chains. Unexpectedly, the thymus-independent gut IEL present in ζ/η-deficient mice were found to express TCR–CD3 complexes associated with FcεRI γ homodimers and therefore appear only moderately affected by the ζ/η mutation. Thus, the expression of FcεRI γ chain-associated TCR isoforms appears able to sustain the maturation of the thymus-independent gut IEL. Moreover, the presence of a faint FcεRI γ band in the TCR–CD3 complexes isolated from normal gut IEL suggests that such TCR isoforms are not unique to the genetically manipulated mice and point to a further degree of complexity in the normal gut IEL population. Finally, the analysis of B cell depleted lymph nodes from ζ/η-deficient mice has allowed

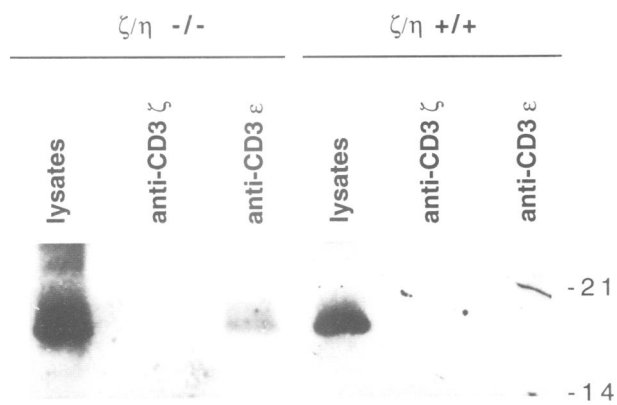

Fig. 11. The TCR–CD3 complexes present on the gut IEL found in ζ/η-deficient mice contain FcεRI γ homodimers. Gut IEL from either a $\zeta/\eta^{-/-}$ mouse or a control littermate ($\zeta/\eta^{+/+}$) were lysed in digitonin lysis buffer solution and immunoprecipitated with hamster monoclonal antibodies directed against CD3-ε (2C11) or CD3-ζ (H146-968). Immunoprecipitates were analyzed on a non-reducing 12% SDS–polyacrylamide gel and immunoblotted with an anti-FcεRI γ serum. Whole-cell lysate (lysates) corresponding to equal cell numbers were processed in parallel to estimate the total amounts of FcεRI γ. The relative positions of the molecular weight standards are indicated (kDa).

us to identify a small subset of $CD4^-CD8^-CD3^+$ cells. However, due to its small size we have not been able to characterize it further and determine for instance if it is made of the previously described large granular lymphocytes (the latter correspond to $CD4^-CD8^-CD3^+$ cells which use FcεRI γ homodimers in lieu of CD3-ζ and CD3-η; Koyasu *et al.*, 1992).

Of particular interest, tumor-bearing mice have been shown to develop $CD8^+$ splenic T cells which are impaired in their function and express FcεRI γ chains instead of CD3-ζ (Mizoguchi *et al.*, 1992). This phenotypic change has been hypothesized to alter the transduction capability of the TCR complexes and accordingly to contribute to the defective immune responses observed in these mice. By forcing the expression of an FcεRI γ transgene into the thymocytes of ζ/η-deficient mice, we should be able to generate mice expressing FcεRI γ associated TCR on the majority of T cells and analyze their functional properties in the absence of ζ polypeptide. Moreover, the reconstitution of $\zeta/\eta^{-/-}$ mice with transgenes encoding functionally impaired ζ subunits should allow us to address directly the role played by the $(YXXL/I)_2$ motifs during intrathymic selection events.

Materials and methods

Vector construction

A mouse CD3-ζ/η genomic clone was isolated from a B10.A genomic phage library (Hue *et al.*, 1990) and mapped by restriction enzyme analysis. A 4.3 kb *Nde*I–*Acc*I fragment containing the exon coding for the transmembrane segment (exon 2, Figure 1A) was isolated from this genomic clone and blunt-end ligated into the *Bam*HI site of plasmid pSP72(*Eco*RV)*Sst*II. The latter was constructed by inserting an *Sst*II linker at the *Eco*RV site of plasmid pSP72 (Promega). A 1.8 kb pgk-neo cassette (neo) derived from plasmid pKJ.1 (McBurney *et al.*, 1991; a gift from M.A.Rudnicki) was subsequently inserted into the *Bam*HI site located within exon 2. Finally, a 1.9 kb herpes simplex thymidine kinase (TK) cassette derived from plasmid pMC1-tk (Mansour *et al.*, 1988; a gift from M.Capecchi) was cloned in the *Sal*I site present in the polylinker located at the 5′ end of the CD3-ζ/η insert. The resulting targeting construct is denoted pRH15 and depicted in Figure 1A. A mock template (pRH17, Figure 1A), used as a positive control during PCR analysis (see below), was assembled by extending the 3′ end of the targeting construct with a 1.4 kb *Acc*I–*Bam*HI genomic restriction fragment (Figure 1A).

Transfection and screening of CD3-ζ/η mutant cell clones

The Bruce 4 ES cell line was derived from C57BL/6 mice and was a gift from Colin L.Stewart. Cells were maintained in the presence of leukemia inhibitory factor and grown on a feeder layer of irradiated (3000 rad) primary embryonic fibroblasts. The targeting fragment used in the electroporation was freed from pSP72 sequences by digestion with *Xho*I and *Sst*II. 5×10^7 ES cells were electroporated with a Bio-Rad Gene Pulser (250 V, 500 μF; electrode distance of 0.4 cm) in the presence of 20–40 μg of targeting fragment. The electroporated cells were plated in five 10 cm dishes containing irradiated primary embryonic fibroblasts isolated from transgenic embryos carrying a neomycin resistance gene (Stewart *et al.*, 1987). G418 (300 μg/ml) was added 24 h after electroporation. After 4 days, gancyclovir (2 μM) was added to four of the five plates. Colonies were harvested after 9 days and transferred into 48-well plates containing primary embryonic fibroblasts. Colonies were grown for 3 days and screened for homologous recombinant by PCR (Köntgen *et al.*, 1993).

PCR analysis of mutant ES cell colonies

Pools of eight colonies were resuspended in the following lysis buffer: 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2 mM $MgCl_2$, 0.5% Nonidet P-40, 0.45% Tween-20, 60 μg/ml proteinase K, and successively incubated for 18 h at 56°C and 45 min at 95°C. 10 μl of each lysate was subjected to PCR analysis using two nested pairs of primers (external primers: 5′-CCGCTCCCGATTCGCGCAGCAGCATCGCC-3′ and 5′-GGGCCAGCTCATTCCTCCCACTCA-3′; internal primers: 5-GGCTCTCCGTCTGTGCTGCCGG-3′ and 5′-GGGGTCCTATTCCCCTTCCTCCAG-3′, the positions of which are indicated in Figure 1A (small horizontal arrows). Lysate samples derived from ES cells transfected with the pRH17 mock construct (see above) were used as positive control during PCR analysis. Colonies from PCR-positive pools were individually reanalyzed. CD3-ζ/η mutant clones were confirmed by Southern blot analysis using a 1.4 kb *Acc*I–*Bam*HI genomic fragment containing exon 3 and part of exon 4 as a probe (Figure 1A). Finally, a neo probe was used to ensure that adventitious, non-homologous recombination had not occurred.

Production of CD3-$\zeta/\eta^{-/-}$ mice

Chimeras were generated essentially as described by Bradley (1987). Briefly, ~10 mutant ES cells were injected into BALB/c blastocysts collected at 3.5 days post-coitus. Injected blastocysts were reimplanted into the uteri of pseudopregnant C57BL/6 × CBA/J F1 females. Chimeric progeny were identified by coat color and chimeric males mated to either BALB/c or C57BL/6 females. $\zeta/\eta^{+/-}$ heterozygous mice were intercrossed to produce homozygous $\zeta/\eta^{-/-}$ mice. Screening of mice for the CD3-ζ/η mutation was performed by Southern blot analysis.

Immunoprecipitation and Western blot analysis

The thymocytes analyzed in Figure 1B were lysed with 2% SDS and boiled for 5 min. Proteins were precipitated with acetone for 30 min at 0°C and pelleted by centrifugation for 5 min at 10 000 *g*. The samples were then separated on an 8% reducing SDS–polyacrylamide gel, transferred to nitrocellulose filters, and immunoblotted using a hamster anti-ζ monoclonal antibody raised against a peptide corresponding to amino acids 151–164 (H146-968; Punt *et al.*, 1991), a rabbit anti-ζ/η serum raised against a synthetic peptide corresponding to amino acids 132–144 (Orloff *et al.*, 1990), or a rabbit antiserum raised against a peptide corresponding to the carboxy-terminal 31 amino acids of the mouse CD3-δ polypeptide (Samelson *et al.*, 1986).

IELs from CD3-$\zeta/\eta^{+/+}$ (4.5×10^6 cells) and CD3-$\zeta/\eta^{-/-}$ (9×10^6 cells) were solubilized in digitonin lysis buffer (1% digitonin, 0.1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM triethanolamine, pH 7.8) for 15 min on ice. Aliquots of post-nuclear supernatants were either precipitated in digitonin lysis buffer using 5 μl of affinity-purified hamster anti-CD3-ζ monoclonal antibody (H146-968) or hamster anti-CD3-ε monoclonal antibody (2C11) and 50 μl of a 50% solution of protein A–Sepharose beads (Pharmacia), or not. Samples were then incubated in sample buffer (2% SDS, 10% glycerol, 0.1 M Tris–HCl, pH 6.8, 0.02% bromophenol blue), separated under non-reducing conditions on 12% SDS–polyacrylamide gels, transferred to nitrocellulose, and developed with a rabbit anti-FcεRI γ antiserum (Orloff *et al.*, 1990) using a horseradish peroxidase-labeled goat anti-rabbit antiserum (ICN) and the ECL detection protocol (Amersham).

Northern blot analysis

Northern blot analysis was performed on total RNA as previously described (Malissen *et al.*, 1988). Hybridization probes corresponding to the TCR Cα and TCR Cβ regions were derived as previously described (Letourneur and Malissen, 1989).

Isolation of gut intraepithelial lymphocytes

Gut IEL were isolated as described by Guy-Grand *et al.* (1991).

Antibodies and flow cytometry

5×10^5 cells were stained with saturating levels of antibodies and $5-10 \times 10^3$ gated events were acquired using a Becton-Dickinson FACScan flow cytometer and analyzed with Lysis II software. Forward and side scatters were used to gate out dead cells. Biotinylated, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against CD4 (GK1.5) and CD8 α (53-6.7) were from Becton–Dickinson. Biotinylated, FITC- or PE-conjugated antibodies against CD3-ε (2C11), CD25 (7D4), CD44 (Pgp-1), HSA (J11d), Thy1 (G7), TCR β (H57-597) and TCR δ (GL3) were purchased from Pharmingen. The antibody against CD8 β (H35-17.2, Blanc *et al.*, 1988) was purified and conjugated in our labs. Biotinylated antibodies were revealed with streptavidin-Cy-chrome (Pharmingen) or streptavidin-tricolor (Caltag).

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References

Anderson,J.A., Abraham,K.M., Nakayama,T., Singer,A. and Perlmutter,R.M. (1992) *EMBO J.*, **11**, 4877–4886.

Baniyash,M., Hsu,V.W., Seldin,M.F. and Klausner,R.D. (1989) *J. Biol Chem.*, **264**, 13252–13257.

Bauer,A., McConkey,D.J., Howard,F.D., Clayton,L.K., Novick,D., Koyasu,S. and Reinherz,E.L. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 3842–3846.

Bendelac,A. and Schwartz,R.H. (1991) *Nature*, **353**, 68–71.

Blanc,D., Bron,C., Gabert,J., Letourneur,F., MacDonald,H.R. and Malissen,B. (1988) *Eur. J. Immunol.*, **18**, 613–619.

Borgulya,P., Kishi,H., Uematsu,Y. and von Boehmer,H. (1992) *Cell*, **69**, 529–537.

Bradley,A. (1987) In Robertson,E.J. (ed.), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. IRL Press, Oxford, pp. 113–151.

Chan,A.C., Iwashima,M., Turck,C.W. and Weiss,A. (1992) *Cell*, **71**, 649–662.

Chan,S.H., Cosgrove,D., Waltzinger,C., Benoist,C. and Mathis,D. (1993) *Cell*, **73**, 225–236.

Clayton,L.K., D'Adamio,L., Howard,F.D., Sieh,M., Hussey,R.E., Koyasu,S. and Reinherz,E.L. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5202–5206.

Davis,C.B., Killeen,N., Crooks,M.E.C., Raulet,D. and Littman,D.R. (1993) *Cell*, **73**, 237–247.

Groettrup,M. and von Boehmer,H. (1993) *Eur. J. Immunol.*, **6**, 1393–1396.

Groettrup,M., Baron,A., Griffiths,G., Palacios,R. and von Boehmer,H. (1992) *EMBO J.*, **11**, 2735–2745.

Grusby,M.J., Auchincloss,H., Lee,R., Johnson,R.S., Spencer,J.P., Zijlstra,M., Jaenisch,R., Papaioannou,V.E. and Glimcher,L.H. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3913–3917.

Guy-Grand,D. and Vassalli,P. (1993) *Curr. Opin. Immunol.*, **5**, 247–252.

Guy-Grand,D., Cerf-Bensussan,N., Malissen,B., Malassis-Seris,M., Briottet,C. and Vassalli,P. (1991) *J. Exp. Med.*, **173**, 471–481.

Hermans,M. and Malissen,B. (1993) *Eur. J. Immunol.*, in press.

Hue,I., Trucy,J., McCoy,C., Couez,D., Malissen,B. and Malissen,M. (1990) *J. Immunol.*, **144**, 4410–4419.

Kappes,D.J. and Tonegawa,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10619–10623.

Kelly,K.A. and Scollay,R. (1992) *Eur. J. Immunol.*, **22**, 329–334.

Kishi,H., Borgulya,P., Scott,B., Karjalainen,K., Traunecker,A., Kaufman,J. and von Boehmer,H. (1991) *EMBO J.*, **10**, 93–100.

Köntgen,F., Süss,G., Stewart,C., Steinmetz,M. and Bluethmann,H. (1993) *Int. Immunol.*, **5**, 957–964.

Koyasu,S., D'Adamio,L., Arulanandam,A.R., Abraham,S., Clayton,L.K. and Reinherz,E.L. (1992) *J. Exp. Med.*, **175**, 203–209.

Letourneur,F. and Malissen,B. (1989) *Eur. J. Immunol.*, **19**, 2269–2274.

Levelt,C.N., Ehrfeld,A. and Eichmann,K. (1993) *J. Exp. Med.*, **177**, 707–716.

Malissen,B. and Schmitt-Verhulst,A.-M. (1993) *Curr. Opin. Immunol.*, **5**, 324–333.

Malissen,M., Trucy,J., Letourneur,F., Rebaï,N., Dunn,D.E., Fitch,F.W., Hood,L. and Malissen,B. (1988) *Cell*, **55**, 49–59.

Mallick,C.A., Dudley,E.C., Viney,J.L., Owen,M.J. and Hayday,A.C. (1993) *Cell*, **73**, 513–519.

Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) *Nature*, **336**, 348–352.

McBurney,M.W., Sutherland,L.C., Adra,C.N., Leclair,B., Rudnicki,M.A. and Jardine,K. (1991) *Nucleic Acids Res.*, **19**, 5755–5761.

Mizoguchi,H., O'Shea,J.J., Longo,D.L., Loeffler,C.M., McVicar,D.W. and Ochoa,A.C. (1992) *Science*, **258**, 1795–1798.

Mombaerts,P., Clarke,A.R., Rudnicki,M.A., Iacomini,J., Itohara,S., Lafaille,J.J., Wang,L., Ishikawa,Y., Jaenisch,R., Hooper,M.L. and Tonegawa,S. (1992a) *Nature*, **360**, 225–231.

Mombaerts,P., Iacomini,J., Johnson,R.S., Herrup,K., Tonegawa,S. and Papaioannou,V.E. (1992b) *Cell*, **68**, 869–877.

Nakayama,K and Loh,D.Y. (1992) *Science*, **257**, 94–96.

Nakayama,T., Ueda,Y., Yamada,H., Shores,E.W., Singer,A. and June,C.H. (1992) *Science*, **257**, 96–99.

Ohno,H. and Saito,T. (1990) *Int. Immunol.*, **2**, 1117–1119.

Orloff,D.G., Ra,C.S., Frank,S.J., Klausner,R.D. and Kinet,J.P. (1990) *Nature*, **347**, 189–191.

Ossendorp,F., Jacobs,H., van der Horst,G., de Vries,E., Berns,A. and Borst,J. (1992) *J. Immunol.*, **148**, 3714–3722.

Penninger,J., Kishihara,K., Molina,T., Wallace,V.A., Timms,E., Hedrick,S.M. and Mak,T.W. (1993) *Science*, **260**, 358–361.

Philpott,K.L., Viney,J.L., Kay,G., Rastan,S., Gardiner,E.M., Chae,S., Hayday,A.C. and Owen,M.J. (1992) *Science*, **256**, 1448–1452.

Punt,J.A., Kubo,R.T., Saito,T., Finkel,T.H., Kathiresan,S., Blank,K.J. and Hashimoto,Y. (1991) *J. Exp. Med.*, **174**, 775–783.

Ravetch,J.V. and Kinet,J.P. (1991) *Annu. Rev. Immunol.*, **9**, 457–492.

Rocha,B., Vassalli,P. and Guy-Grand,D. (1992) *Immunol. Today*, **13**, 449–454.

Rodewald,H.R., Arulanandam,A.R., Koyasu,S. and Reinherz,E.L. (1991) *J. Biol Chem.*, **266**, 15974–15978.

Samelson,L.E. and Klausner,R.D. (1992) *J. Biol Chem.*, **267**, 24913–24916.

Samelson,L.E., Weissman,A.M., Robey,F.A., Berkower,I. and Klausner,R.D. (1986) *J. Immunol.*, **137**, 3254–3258.

Seth,A., Gote,L., Nagarkatti,M. and Nagarkatti,P.S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7877–7881.

Shinkai,Y., Rathbun,G., Lam,K.P., Oltz,E.M., Stewart,V., Mendelsohn,M., Charron,J., Datta,M., Young,F., Stall,A.M. and Alt,F.W. (1992) *Cell*, **68**, 855–867.

Shinkai,Y., Koyasu,S., Nakayama,K.-I., Murphy,K.M., Loh,D.Y., Reinherz,E.L. and Alt,F.W. (1993) *Science*, **259**, 822–825.

Shores,E.W., Nakayama,T., Wiest,D.L., Takahama,Y., Sharrow,S. and Singer,A. (1993) *J. Immunol.*, **150**, 1263–1275.

Shortman,K. (1992) *Curr. Opin. Immunol.*, **4**, 140–146.

Shortman,K., Egerton,M., Spangrude,G.J. and Scollay,R. (1990) *Semin. Immunol.*, **2**, 3–12.

Stewart,C.L., Schuetze,S., Vanek,M. and Wagner,E.F. (1987) *EMBO J.*, **6**, 383–388.

Swat,W., Dessing,M., Baron,A., Kisielow,P. and von Boehmer,H. (1992) *Eur. J. Immunol.*, **22**, 2367–2372.

Takahama,Y. and Singer,A. (1992) *Science*, **258**, 1456–1462.

Takahama,Y., Shores,E.W. and Singer,A. (1992) *Science*, **258**, 653–656.

Torres-Nagel,N., Kraux,E., Brown,M.H., Tiefenthaler,G., Mitnacht,R., Williams,A. and Hunig,T. (1992) *Eur. J. Immunol.*, **22**, 2841–2848.

Turka,L.A., Schatz,D.G., Oettinger,M.A., Chun,J.J., Gorka,C., Lee,K., McCormack,W.T. and Thompson,C.B. (1991) *Science*, **253**, 778–781.

von Boehmer,H. and Kisielow,P. (1993) *Cell*, **73**, 207–208.

Wegener,A.-M.K., Letourneur,F., Hoeveler,A., Brocker,T., Luton,F. and Malissen,B. (1992) *Cell*, **68**, 83–95.

Weiss,A. (1993) *Cell*, **73**, 209–212.

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