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Abnormal T cell development in CD3-$\zeta^{-/-}$ mutant mice and identification of a novel T cell population in the intestine

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The T cell antigen receptor (TCR)-associated invariable membrane proteins (CD3-γ, -δ, -ϵ and -ζ) are critical to the assembly and cell surface expression of the TCR/CD3 complex and to signal transduction upon engagement of TCR with antigen. Disruption of the CD3-ζ gene by homologous recombination resulted in a structurally abnormal thymus which primarily contained $CD4^-CD8^-$ and TCR/$CD3^{very\ low}CD4^+CD8^+$ cells. Spleen and lymph nodes of CD3-$\zeta^{-/-}$ mutant mice contained a normal number and ratio of $CD4^+$ and $CD8^+$ single positive cells that were TCR/$CD3^{very\ low}$. These splenocytes did not respond to antibody cross-linking or mitogenic triggering. The Vβ genes of $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes and splenic T cells were productively rearranged. These data demonstrated that (i) in the absence of the CD3-ζ chain, the $CD4^-CD8^-$ thymocytes could differentiate to $CD4^+CD8^+$TCR/$CD3^{very\ low}$ thymocytes, (ii) that thymic selection might have occurred, (iii) but that the transition to $CD4^+CD8^-$ and $CD4^-CD8^+$ cells took place at a very low rate. Most strikingly, intraepithelial lymphocytes (IELs) isolated from the small intestine or the colon expressed normal levels of TCR/CD3 complexes on their surface which contained FcϵRIγ homodimers. In contrast to CD3-ζ containing IELs, these cells failed to proliferate after triggering with antibody cross-linking or mitogen. In comparison to thymus-derived peripheral T cells in the spleen and lymph nodes, the preferential expression of normal levels of TCR/CD3 in intestinal IELs suggested they mature via an independent extrathymic pathway.

Key words: CD3/gene targeting/intraepithelial lymphocyte/T cell development/TCR

Introduction

During T cell development, pre-thymic stem cells that derive from the bone marrow in adult mice, or fetal liver or the yolk sac in the embryo (Liu and Auerbach, 1991a,b; Ikuta *et al.*, 1992), enter the thymus where they undergo extensive proliferation and differentiation in a process of thymic education (Marrack and Kappler, 1987; von Boehmer, 1990). Although it is known that the thymus plays a central role in the maturation of T cells from pre-thymic stem cells, extrathymic differentiation of T cells may occur, e.g. in the intestine and in aged nude mice (MacDonald *et al.*, 1987; Mosley and Klein, 1992; Poussier *et al.*, 1992; Rocha *et al.*, 1992).

Various surface markers may be used to divide thymocytes into phenotypically distinct stages of differentiation. These markers include, but are not restricted to: TCR (T cell antigen receptor)/CD3, CD4, CD8, Thy1, HSAg (heat stable antigen), IL2Rα and Ly6 (reviewed in Petrie *et al.*, 1990; von Boehmer, 1990; Ikuta *et al.*, 1992). Among CD4/CD8 defined subsets, intrathymic precursors are found within the $CD4^-CD8^-$ double negative (DN) population and are also negative for TCR/CD3. These DN precursors can differentiate to $CD4^+CD8^+$ double positive (DP) cells via TCR/$CD3^-CD4^-CD8^+$ or $CD4^+CD8^-$ intermediates and then become mature $CD4^+$ or $CD8^+$ single positive (SP) cells expressing TCR/CD3. The TCR/CD3 complex is the most specific mature T cell marker and the major cell surface structure responsible for antigen specificity. Its proper expression is essential to normal T cell development as demonstrated recently in various TCRα- and β-deficient mice (Bosma and Carrol, 1991; Mombaerts *et al.*, 1992a,b; Philpott *et al.*, 1992; Shinkai *et al.*, 1992).

The TCR/CD3 complex is composed of at least six distinct subsets (Clevers *et al.*, 1988; Ashwell and Klausner, 1990). The highly polymorphic TCR chains consist of either $\alpha-\beta$ or $\gamma-\delta$ disulfide-linked heterodimers with short cytoplasmic tails and determine antigenic specificity. The CD3 molecules, including the γ, δ, ϵ and ζ proteins, are invariant chains with relatively large intracytoplasmic domains which associate noncovalently with the TCR heterodimers. In murine T cells, a receptor-associated η chain can be produced through alternative splicing of the ζ mRNA transcript (Jin *et al.*, 1990), but its presence in human T cells remains to be demonstrated. The CD3 invariant chains are essential to the intracellular assembly and surface expression of the complete TCR/CD3 complex, and are responsible for coupling TCR antigen recognition to intracellular signal transduction pathways.

All of the TCR/CD3 complex subunits are transmembrane proteins. They predominantly associate with a minimum stoichiometry of $\alpha\beta$ $\gamma\delta\epsilon\epsilon$ $\zeta\zeta$, but they may display other subunit combinations (Blumberg *et al.*, 1990). Recent evidence suggests that a divalent TCR/CD3 complex: may exist as $(\alpha\beta)_2$ $\gamma\delta\epsilon_2$ ζ_2 (Alarcon *et al.*, 1991; Exley *et al.*, 1991; Terhorst and Regueiro, 1993). The CD3-ϵ and -ζ homodimers constitute two distinct modules that are involved in mediating signals generated through engagement of TCRs with antigens (Letourneur and Klausner, 1992; Wegener *et al.*, 1992; Terhorst and Regueiro, 1993).

The γ chain of the high affinity IgE receptor (FcϵRIγ chain), and the CD3-ζ and -η chains are structurally

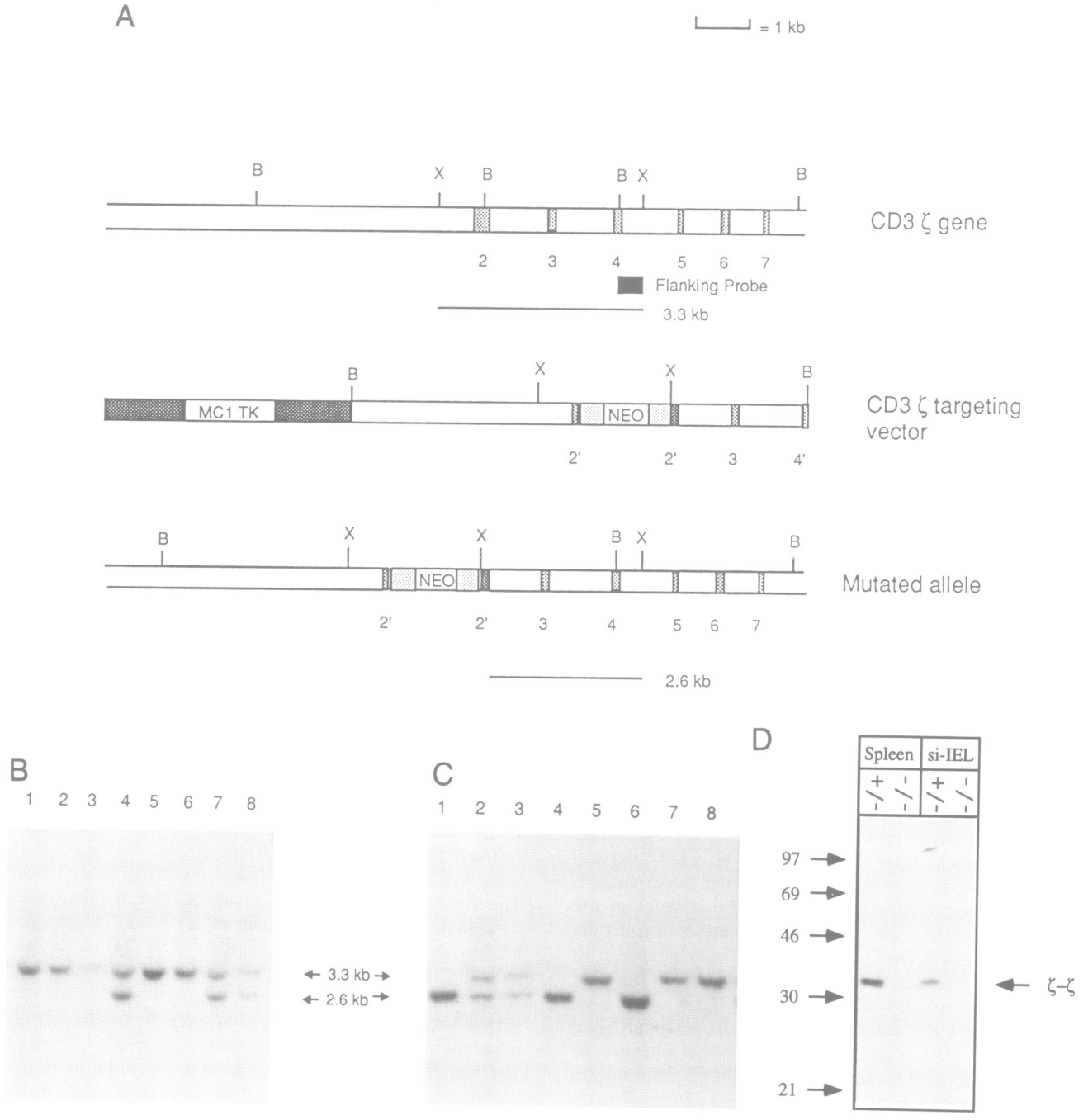

Fig. 1. Disruption of CD3-ζ gene via homologous recombination and Southern and Western analysis of targeted cells and mutant mice. (**A**) Diagram of targeting vector for homologous recombination. Numbers 2–7 represent exons of CD3-ζ gene. 2′ and 4′ represent portions of exons 2 and 4, respectively. Abbreviations for restriction enzyme sites: B, *Bam*HI; X, *Xba*I. (**B**) Southern blot of ES cell clone DNAs. *Xba*I digests of DNA were hybridized with the flanking probe indicated in panel A. Lanes 4, 7 and 8 contain targeted events. The mutated allele is at 2.6 kb and wild type allele is at 3.3 kb. (**C**) Southern blot analysis of tail DNA from mouse littermates from a cross between CD3-ζ heterozygotes. Lanes 1, 4 and 6 represent mice homozygous for the mutation; lanes 2 and 3 are heterozygotes; lanes 5, 7 and 8 are wild type. (**D**) Western blot analysis of $\zeta^{-/-}$ and $\zeta^{-/+}$ mutant mice showing the absence of the ζ chain protein in spleen and intestinal intraepithelial cells. Whole cell lysates were blotted with an anti-ζ monoclonal antibody, 1C10.1. Molecular weight standards are indicated in kDa.

homologous proteins that may form disulfide-linked homo and heterodimers (e.g. $\gamma-\gamma$, $\zeta-\gamma$ or $\eta-\gamma$) and associate with other TCR/CD3 chains in T cells (Ra *et al.*, 1989b; Orloff *et al.*, 1990; Ravetch and Kinet, 1991). In addition to T cells, the FcεRIγ chain homodimer can be expressed in basophils, mast cells, macrophages and natural killer (NK) cells, and associates with FcεRI and FcγRIII (CD16) chains (Ra *et al.*, 1989b; Ravetch and Kinet, 1991). In human NK cells, the low affinity Fc receptor (FcR) for IgG (CD16) associates with disulfide-linked ζ or FcεRIγ chains (Anderson *et al.*, 1989, 1990; Lanier *et al.*, 1989). On the other hand, the association of the ζ chain with other FcR chains has not been documented in mice.

The ζ and the FcεRIγ chains are critical to both the cell surface expression and functional integrity of the TCR/CD3 or the FcR complexes respectively (Bonifacino *et al.*, 1990;

Ra *et al.*, 1989a,b; Weissman *et al.*, 1989; Ravetch and Kinet, 1991; Rodewald *et al.*, 1991; Romeo and Seed, 1991). Activation of T cells by either antigens or anti-CD3 antibody induces the tyrosine phosphorylation of ζ chain. Moreover, the requirement for the intracytoplasmic domain of the ζ chain in triggering T cell activation responses has been demonstrated *in vitro* using a series of truncation mutants (Frank *et al.*, 1990; Wegener *et al.*, 1992). Cross-linking of the FcεRI chain likewise results in tyrosine phosphorylation of the FcεRIγ chain (Paolini *et al.*, 1991). Functional similarities between the ζ and FcεRIγ chains have been shown by (i) their ability to substitute for one another in the reconstitution of either the FcR or TCR/CD3 complexes *in vitro* (Lanier *et al.*, 1991; Rodewald *et al.*, 1991); and (ii) the fact that chimeric molecules containing CD4 extracellular domains fused to the FcεRIγ chain cytoplasmic tail can at least partially substitute for ζ and η cytoplasmic tails in T cell activation assays (Romeo and Seed, 1991).

Although the function of the ζ chain in T and NK cells has been studied extensively *in vitro*, its role *in vivo* remains to be determined. In this report, we have used gene targeting in mouse embryonic stem (ES) cells to disrupt the expression of the ζ chain gene in the germline of mice. Mice homozygous for the mutation in the ζ chain gene have abnormal T cell development manifested not only by the accumulation of relatively immature thymocytes, but also by the expression of very low levels of TCR/CD3 on the cell surface of thymocytes and splenic T cells. Most strikingly, we found that intestinal intraepithelial lymphocytes (IELs) can express normal levels of TCR/CD3 on the cell surface. Since the ζ chain was not detected in these IELs and was replaced by the FcεRIγ chain, we have identified a novel T cell population that is predominantly found in the intestine and which differs from other peripheral T cells present in spleen and lymph node.

Results

Generation of CD3-ζ$^{-/-}$ mice

The murine gene for CD3-ζ consists of eight exons spanning over 24 kb, with a first intron of >20 kb (Baniyash *et al.*, 1989). To disrupt CD3-ζ gene expression, a targeting vector was made by incorporating an expression cassette for neomycin resistance (PGKneo) into the middle of exon 2. The targeting vector (Figure 1A) was constructed using two contiguous genomic fragments of 4.2 and 2.3 kb. A synthetic linker containing translation stop codons in three reading frames and the PGKneo cassette were inserted between the two fragments at the *Bam*HI site, which divides exon 2. To select against random insertion events in ES cells, the HSV (herpes simplex virus) thymidine kinase expression cassette, MC1TK, was added to the 5′-end of the 4.2 kb *Bam*HI fragment.

Linearized targeting vector DNA was introduced by electroporation into the AB-1 ES cell line (McMahon and Bradley, 1990) and clones were doubly selected in G418 and FIAU. Identification of targeted clones was made by Southern blot analysis of ES cell genomic DNA digested with *Xba*I and hybridized with a probe flanking the 3′ junction of recombination (flanking probe in Figure 1A). Approximately one in seven G418/FIAU selected clones contained targeted events as shown by the presence of the predicted 2.6 kb band compared with the 3.3 kb wild type band (Figure 1B). Five separate ES cell clones were injected into C57BL/6 blastocysts to generate chimeric mice. Founders from all five lines led to germline transmission of the mutation, as determined by Southern blot analysis of tail genomic DNA in the same fashion shown for ES cell clones. Animals heterozygous for the mutation were crossed to derive offspring homozygous for the mutant gene. An example of Southern blot analysis of germline animal DNAs is shown in Figure 1C. The complete disruption of CD3-ζ gene expression was demonstrated by the absence of protein in T cells. The latter was shown by Western blot analysis using the anti-ζ monoclonal antibody 1C10.1 which did not detect CD3-ζ protein in either splenocytes or intestinal IELs (Figure 1D). Thus, a mutant mouse had been generated with a disruption in the CD3-ζ gene.

Abnormal T cell development in the thymus of CD3-ζ$^{-/-}$ mice

We examined the effects of the CD3-ζ mutation on thymic structure and T cell differentiation by immunohistology and cell surface phenotyping. Both the size and the cellular content of the thymus from ζ$^{-/-}$ adult mice were significantly reduced relative to those of control wild type or ζ$^{-/+}$ mice. Thymuses from mutant mice at the age of 8–12 weeks contained 5- to 10-fold fewer thymocytes than wild type. When examined by immunohistochemistry using Thy1 antibody, the mutant thymus appeared to have an abnormal architecture which consisted almost entirely of cortex, with only very small regions identified as medullae (data not shown). This result suggested that most thymocytes were Thy1$^+$ immature cortical thymocytes.

Thymocyte development in ζ$^{-/-}$ mice was severely affected at the stage where CD4$^-$CD8$^-$ DN cells differentiate to CD4$^+$CD8$^+$ DP cells and thereafter to either CD4$^+$ or CD8$^+$ SP cells. First, cells coexpressing Thy1$^+$ and TCR/CD3^{hi} were undetectable in mutant mice (Figure 2A). However, these Thy1$^+$ cells did show a very low level of anti-CD3 and anti-TCR staining (TCR/CD3$^{very\ low}$). When compared with control mice it represented even fewer TCR/CD3 complexes than normally found on TCR/CD3^{low} thymocytes (Figure 2B). Second, staining with anti-CD4 and anti-CD8 antibodies revealed an increase in the percentage of CD4$^-$/CD8$^-$ DN cells, and decreases in the percentages of CD4$^+$/CD8$^+$ DP cells and SP cells (Figure 2A). However, the absolute number of CD4$^-$/CD8$^-$ DN thymocytes was comparable with that of normal mice suggesting that the presence of the ζ chain was critical to the differentiation of CD4$^-$/CD8$^-$ DN to TCR/CD3^{hi} CD4$^+$/CD8$^+$ DP cells.

The relatively immature phenotype of thymocytes was further demonstrated by the higher percentages of cells expressing HSAg^{hi} (heat stable antigen) (45%), IL2Rα$^+$ (interleukin 2 receptor) (46%) and Ly6A/E$^+$ (28.5%) compared with those of control mice (<2% of each marker) (Figure 2C). These molecules are expressed on immature thymocytes during early stages of development (Petrie *et al.*, 1990; Ikuta *et al.*, 1992).

Taken together, the phenotypic studies show that the absence of ζ chain retarded the differentiation of CD4$^-$/CD8$^-$ DN to TCR/CD3^{hi} CD4$^+$/CD8$^+$ DP cells, reduced the expression of normal levels of TCR/CD3 and thus impaired the normal development of thymocytes and thymic structure.

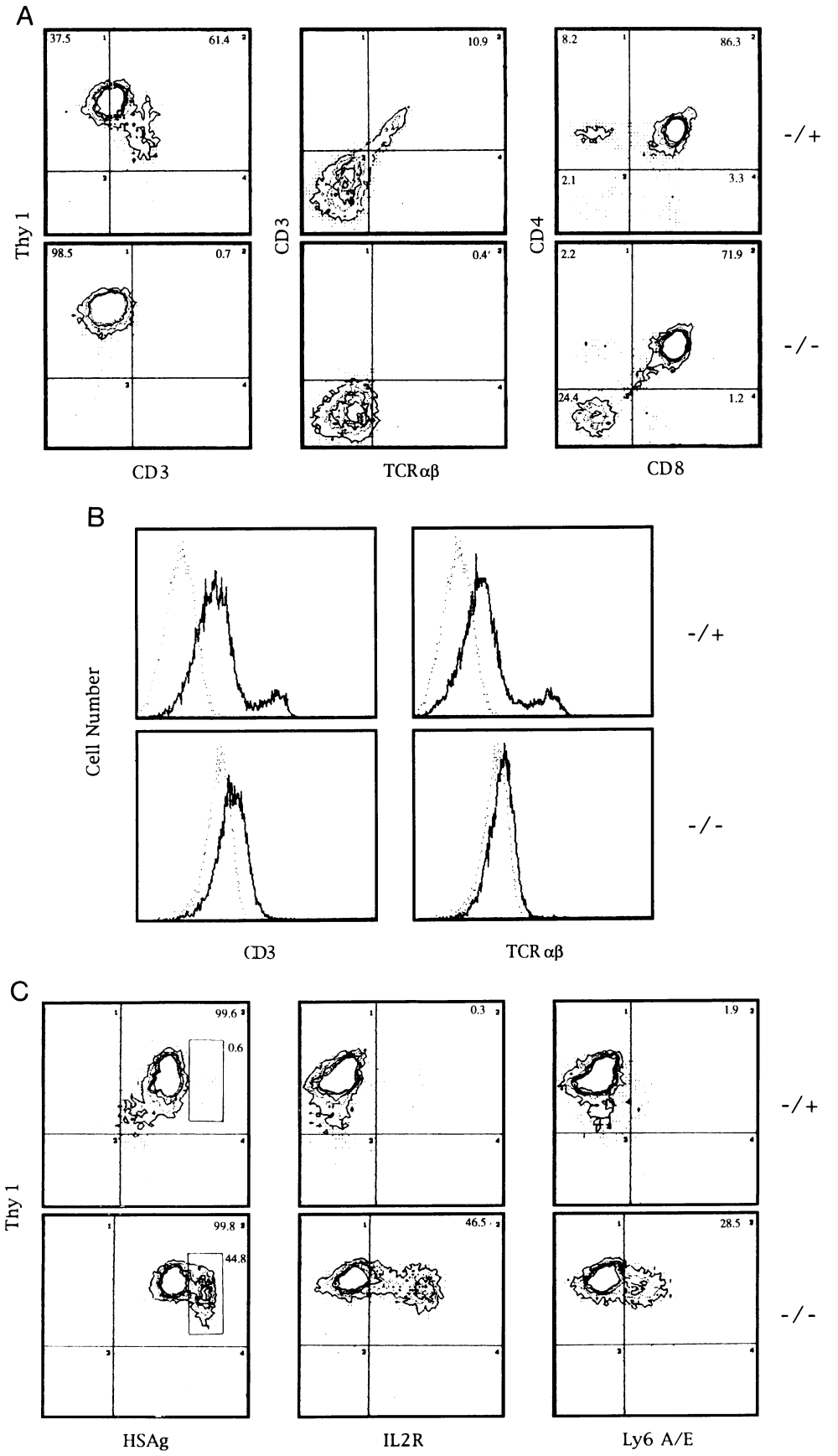

Fig. 2. Flow cytometry analysis of thymocytes obtained from $\zeta^{-/-}$ mutant mice and normal $\zeta^{-/+}$ control mice. (**A**) Two color staining analysis of Thy1, CD3, TCRα/β, CD4 and CD8 expression showing no CD3/TCR^{hi} thymocytes and the presence of a higher percentage DN thymocytes in mutant mice. (**B**) Single color staining analysis of CD3 and TCRα/β expression. (**C**) Two color staining analysis of HSAg, IL2R and Lys6A/E expression on Thy1^{+} thymocytes showing the presence of more immature thymocytes in the mutant mouse.

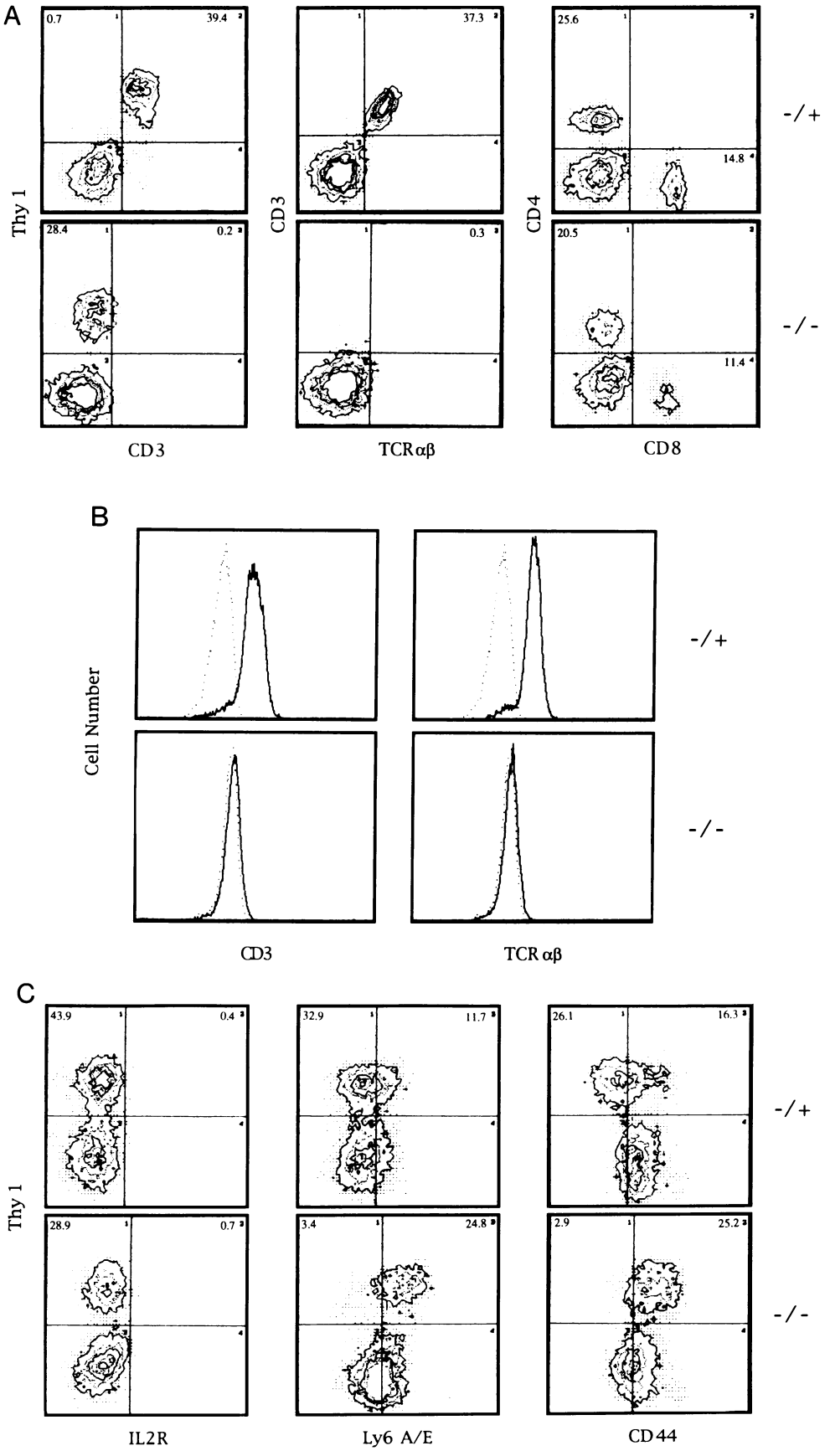

Fig. 3. Flow cytometry analysis of splenocytes obtained from $\zeta^{-/-}$ mutant mice and normal $\zeta^{-/+}$ control mice. (**A**) Two color staining analysis of Thy1, CD3, TCRα/β, CD4 and CD8 expression showing no CD3/TCR^{hi} cells but a normal ratio of CD4$^+$ to CD8$^+$ SP cells in mutant mice. (**B**) Single color staining analysis showing very low levels of CD3 and TCRα/β expression on splenocytes. The splenocytes used in the flow cytometry staining were T cells purified using a T cell affinity column as described in Materials and methods. (**C**) Two color staining analysis of IL2R, Ly6A/E and CD44 expression on Thy1$^+$ cells.

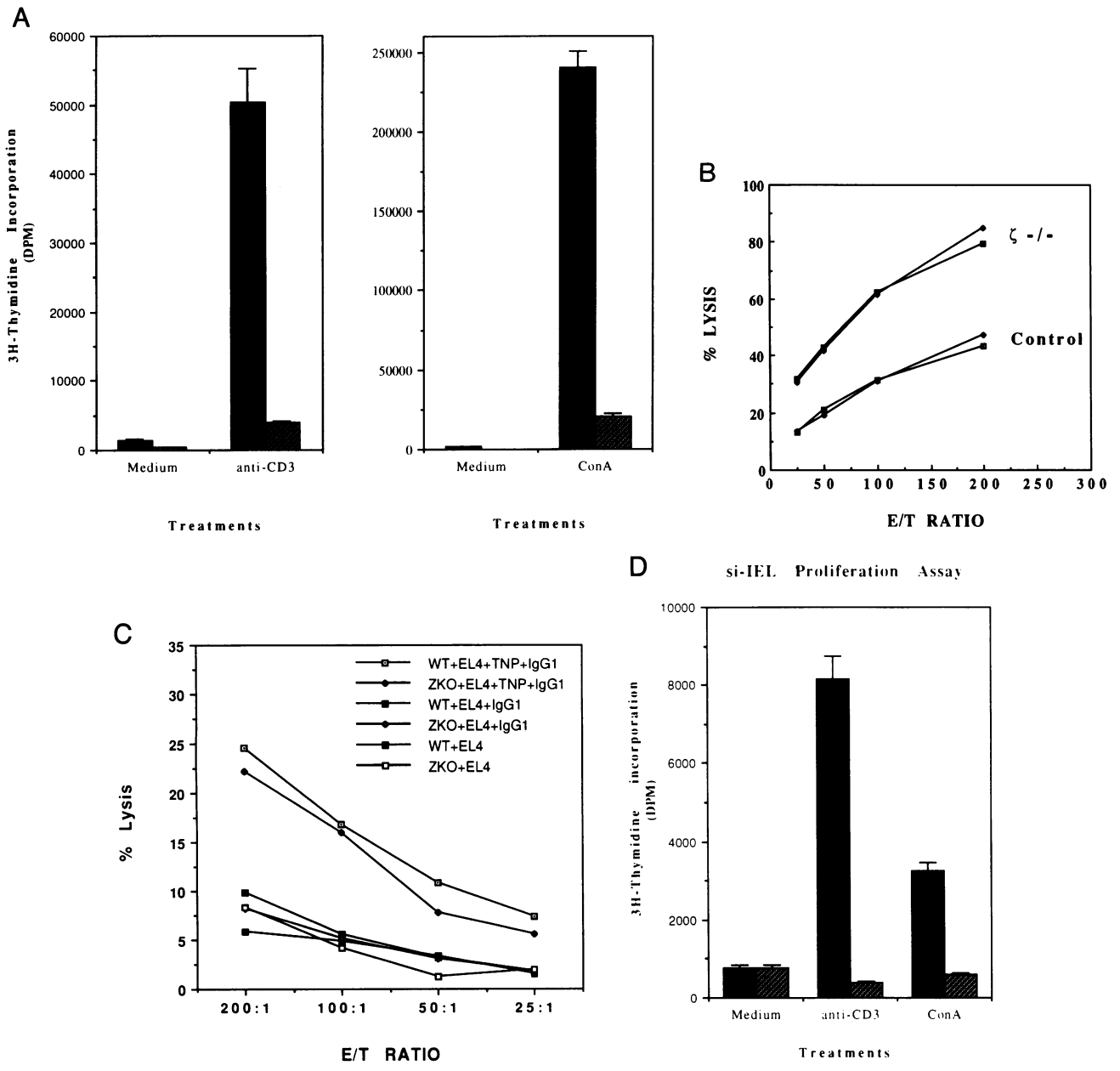

Fig. 4. Functional analyses of spleen cells and intraepithelial lymphocytes isolated from the small intestine of CD3-$\zeta^{-/-}$ or CD3-$\zeta^{-/+}$ mice. (**A**) Proliferative responses of splenic T cells to anti-CD3 and ConA. ■, −/+; ▨, −/−. (**B**) Natural killer cell activity of splenocytes against YAC-1 tumor cell targets. (**C**) ADCC assay of splenocytes (the results shown in B and C are representative of three different experiments). (**D**) Proliferative responses of small intestinal IELs to anti-CD3 and ConA. ▩, −/+; ▨, −/−.

TCR/CD3$^{very\ low}$CD4$^+$ or CD8$^+$ T cells are present in the spleen and lymph nodes

Unlike the thymus, the spleen and the lymph nodes of $\zeta^{-/-}$ mutant mice were relatively larger than those in control animals, but they contained normal levels of T cells. Similar to thymocytes, splenic T cells expressed barely detectable levels of TCR/CD3 complexes by flow cytometry analysis (Figure 3A and B). However, despite the expression of very low levels of TCR/CD3, the ratio of CD4$^+$ to CD8$^+$ SP cells was normal (Figure 3A). These cells were negative for IL2Rα, suggesting that they were more mature cells than IL2Rα^+ thymocytes (Figure 3C), but they expressed higher levels of Ly6 A/E and CD44 relative to controls (Figure 3C). Although the roles of Ly6 A/E and CD44 molecules in T cell development remain unclear, they may participate in the proliferation and expansion of splenic T cells derived from thymocytes.

Therefore, a relatively normal number and ratio of CD4$^+$ and CD8$^+$ SP cells were present in the spleen and lymph nodes despite the severe effect of the ζ mutation on thymocyte differentiation. This suggests that the exit from the thymus and the expansion in the peripheral lymphoid organs of SP cells is not strictly dependent on the inclusion of ζ in the TCR/CD3 complex or upon normal expression levels of the complex.

Analyses of T cell and NK cell functions

Normal functional T cells can proliferate in response to antigen recognition or mitogen stimulation. Although development of the T cell lineage was abnormal in $\zeta^{-/-}$ mice

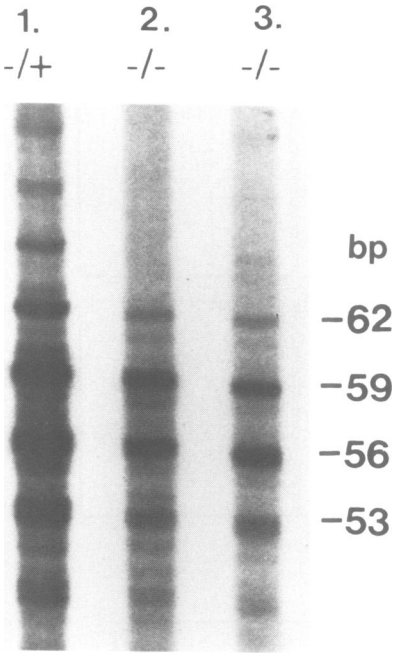

Fig. 5. Analysis of TCRβ gene segment rearrangement. Genomic DNA was extracted from thymocyte subsets isolated by fluorescence activated cell sorting. PCR amplification of DNA was performed using Vβ4- and Jβ2.2-specific oligonucleotide primers. Polyacrylamide gel electrophoresis of ^{32}P-labeled PCR products is shown for the following cell types: lane 1, $CD8^-$ thymocytes from control $\zeta^{-/+}$ mice; lane 2, $CD4^+/CD8^+$ DP thymocytes from $\zeta^{-/-}$ mice; lane 3, $CD4^-/CD8^-$ DN thymocytes from $\zeta^{-/-}$ mice. Fragment lengths are listed in base pairs (bp). Similar results showing productive rearrangement band pattern were obtained using primers specific for Vβ4 and Jβ2.5 gene segments (not shown).

based on phenotypic studies, it was not clear whether the TCR/CD3$^{\text{very low}}$ $CD4^+$ and $CD8^+$ SP cells in the periphery could be activated through the antigen receptor. The functional competence of T cells of $\zeta^{-/-}$ mice was examined by their responses to anti-CD3 and ConA. Proliferative responses of enriched splenic T cells to either anti-CD3 or ConA were slightly, if at all, above background (Figure 4A). These very weak responses may have been due to the presence of very low levels of TCR/CD3 complexes. Treatment with phorbol 12-myristate 13-acetate (PMA) and ionomycin determined that the block in activation was proximal to the T cell receptor (data not shown).

In human NK cells the ζ chain protein associates with CD16 (FcγRIII). Since this association in mouse cells has not been established, the effect of the ζ mutation on NK cell function was examined. Our results showed that the $\zeta^{-/-}$ mutant mice possessed relatively normal to elevated NK cell activity against YAC-1 target cells (Figure 4B) and in assays for antibody-dependent cellular cytotoxicity (ADCC) (Figure 4C). Therefore, the ζ protein is not indispensable to the function of murine NK cells.

These results indicate that functionally inert T cells and normal NK cells were present in the peripheral lymphoid tissues of the ζ mutant mice.

Productively rearranged Vβ–Jβ genes are present in $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes, and in splenic T cells

Mutations that affect the rearrangement of TCR genes, i.e. SCID, RAG-1 and RAG-2, halt the progression of $CD4^-/CD8^-$ DN to $CD4^+/CD8^+$ DP cells in the thymus (Bosma and Carrol, 1991; Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992). This effect is specifically found in $TCR\beta^{-/-}$ mutant mice, but not in $TCR\alpha^{-/-}$ mutant mice (Mombaerts *et al.*, 1992b; Philpott *et al.*, 1992). The expression of a TCRβ transgene in RAG-2 mutant mice restores the development of $CD4^+/CD8^+$ DP cells which were also shown to express low levels of a TCR/CD3 complex that lacked the ζ homodimer (Shinkai *et al.*, 1993). Moreover, a recent study has shown that productive rearrangements of the Vβ chain gene occur non-stochastically in the majority of immature thymocytes and results in an enrichment of productively rearranged cells (Mallick *et al.*, 1993). Together, these observations imply that the transition of $CD4^-/CD8^-$ DN to $CD4^+/CD8^+$ DP cells in the thymus is regulated mainly by the productive rearrangement of TCRβ chain genes.

We performed a population analysis to examine Vβ to Jβ gene rearrangements in $CD4^-/CD8^+$ DN and $CD4^+/CD8^+$ DP thymocytes, and splenocytes from ζ mutant mice. Oligonucleotide primers complementary to conserved portions of the Vβ and Jβ regions were used to characterize TCR genomic variable region sequences by PCR amplification. Since random N-region additions of TCR genes are expected to be out-of-frame (and thus non-productive) in two out of three instances, a given population of TCR gene alleles would show a non-uniform length banding pattern. However, Mallick *et al.* (1993) have shown that even relatively immature thymocyte populations are enriched for cells that contain a productively rearranged TCRβ chain gene allele. Thus, ~75% of TCRβ chain gene variable region sequences from these thymocytes are in-frame and differ by multiples of 3 bp. Our analysis of Vβ4 to Jβ2.2 (Figure 5) and to Jβ2.5 (not shown) joining indicated that there were predominantly productive TCRβ chain gene rearrangements in $CD4^-/CD8^-$ DN and $CD4^+/CD8^+$ DP thymocytes and in splenocytes from mutant mice.

These results show that the cells are being enriched for productive Vβ chain gene rearrangements prior to becoming $CD4^+/CD8^+$ DP thymocytes, probably at some stage of $CD4^-/CD8^-$ DN development. Furthermore, the ζ chain was not essential for this process since the $\zeta^{-/-}$ mutant mice showed no differences in their banding pattern from the control.

Normal level of TCR/CD3 cell surface expression in IELs due to assembly with FcεRIγ homodimer

The intestine is considered a first-line lymphoid tissue that can respond to various foreign antigens and may also provide a thymus-like environment for hematopoeitic stem cells to differentiate into mature T cells extrathymically (Lefrançois, 1991; Rocha *et al.*, 1991, 1992; Mosley and Klein, 1992; Poussier *et al.*, 1992). IELs isolated from the gastrointestinal tract of normal mice contain a relatively high percentage of $CD8^+$ compared with $CD4^+$ SP cells, and a small but distinguishable percentage of $CD4^+CD8^+$ T cells. In addition, intestinal IELs contain a unique population that expresses CD8αα homodimers rather than the CD8αβ heterodimers characteristic of other peripheral T cells.

The CD4/CD8 defined subsets in IELs isolated from the small intestine (si-IEL) and large intestine (li-IEL) of $\zeta^{-/-}$ mice were examined by flow cytometry. There were higher ratios of $CD8^+$ to $CD4^+$ SP cells in both populations, consistent with the ratios seen in control mice (Figure 6A).

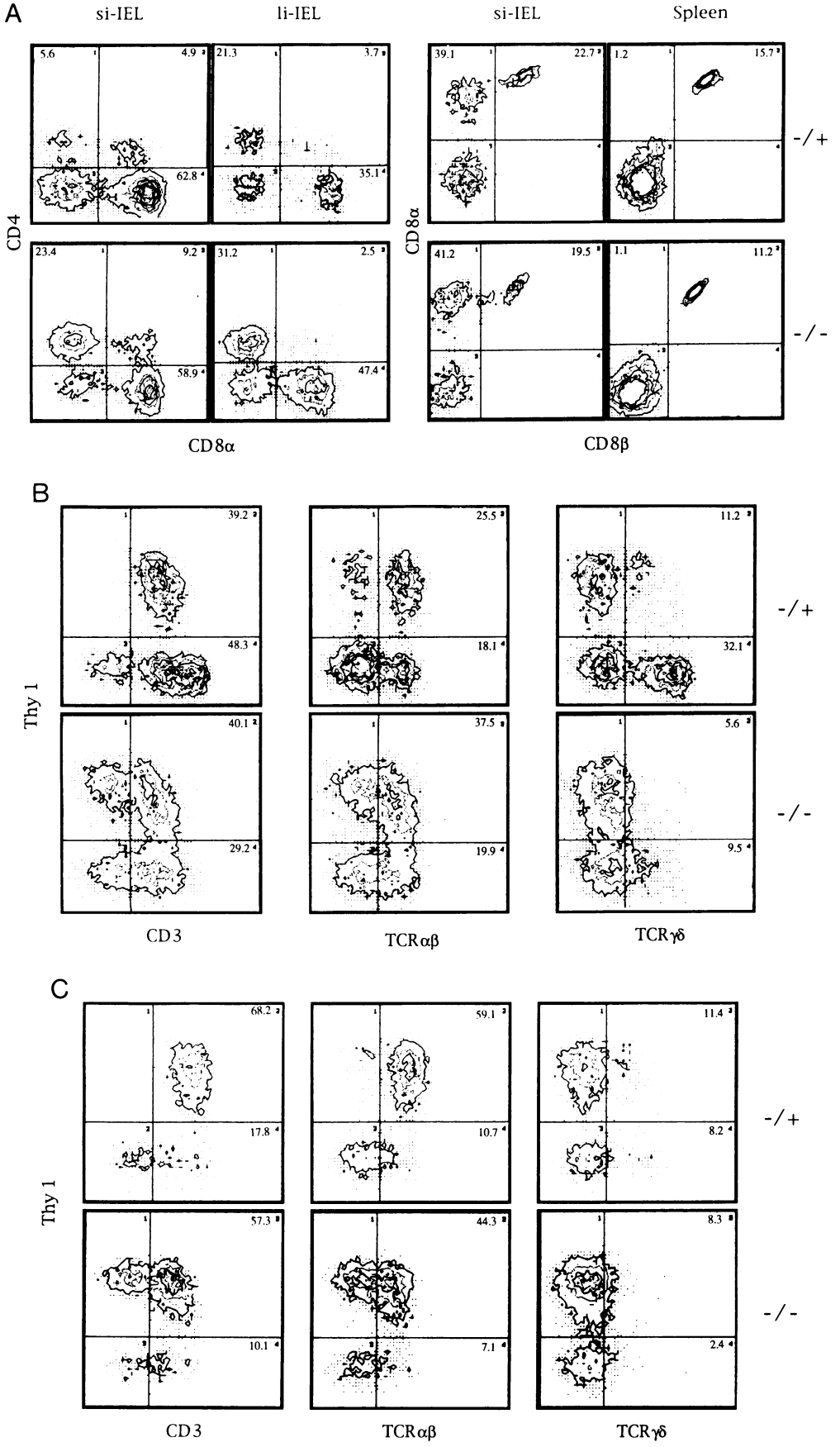

Fig. 6. Flow cytometry analysis of small intestinal and large intestinal IELs obtained from $\zeta^{-/-}$ mutant mice and $\zeta^{-/+}$ control mice. (**A**) Two color staining analysis of CD4/CD8α and CD8α/CD8β expression. (**B**) Two color staining analysis of Thy1 and either CD3, TCRα/β or TCRγ/δ expression on small intestinal IELs. (**C**) Two color staining analysis of Thy1 and either CD3, TCRα/β or TCRγ/δ expression on large intestinal IELs.

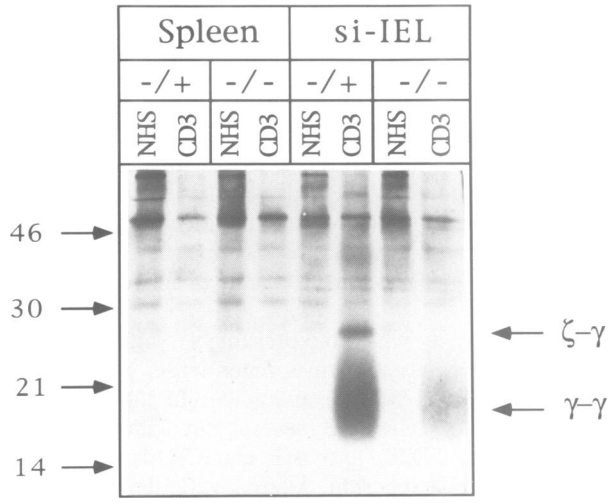

Fig. 7. Association of FcεRIγ chain with other TCR/CD3 chains in intestinal IELs. Western blot analysis of splenic T cells and small intestinal IELs. Cell lysates were immunoprecipitated with anti-CD3-ε antibody and blotted with anti-FcεRIγ antiserum. The positions of proteins with apparent molecular weights of ζ−FcεRIγ heterodimers and FcεRIγ homodimers are indicated on the right. Protein molecular weight size standards are indicated on the left in kDa. NHS, normal hamster serum.

Furthermore, ~60% of $CD8^+$ si-IELs expressed $CD8\alpha\alpha$ homodimers rather than $CD8\alpha\beta$ heterodimers. $CD4^+CD8^+$ DP cells were also present in IELs isolated from both $\zeta^{-/-}$ and $\zeta^{-/+}$ mice. Thus the ζ muation had little effect on the relative distribution of $CD8^+$ and $CD4^+$ SP cells in the intestine.

In striking contrast to other peripheral T cells, the majority of both si-IELs and li-IELs from $\zeta^{-/-}$ mutant mice expressed relatively normal levels of TCR/CD3 molecules (Figure 6B and C). But unlike control mice, not all $Thy1^+$ cells were $TCR/CD3^+$. Although these $TCR/CD3^-$ cells could have been IELs, it was possible that they represented lamina propria lymphocytes which, similar to other thymus-derived peripheral T cells, might not express normal levels of TCR/CD3 due to the ζ mutation. Interestingly, both $Thy1^+$ and $Thy1^-$ intestinal IELs from $\zeta^{-/-}$ mice displayed primarily $TCR\alpha\beta$ rather than the $TCR\gamma\delta$ molecules found in control mice. This skewing was less pronounced in the IELs obtained from one mouse >6 months of age, which showed nearly a one to one ratio of $TCR\gamma\delta^+$ to $TCR\alpha\beta^+$ cells (data not shown). This suggests that the amount of $TCR\gamma\delta$-expressing cells may vary with age in the intestine of $\zeta^{-/-}$ mice.

Previous studies showed that the proliferative responses of normal mouse IELs to T cell mitogens were much weaker than those of splenic T cells (Mowat *et al.*, 1986, 1989; Mosley *et al.*, 1991; Barrett *et al.*, 1992; Gramzinski *et al.*, 1993). Since the si-IELs expressed a normal level of TCR/CD3 on their cell surface, their proliferative responses to anti-CD3 and ConA were examined. As also demonstrated by others, proliferation of normal intestinal IELs in these assays was low but positive (Figure 4D). In contrast,the IELs of $\zeta^{-/-}$ mutant mice failed to respond to anti-CD3 antibody and the mitogen, ConA, despite their normal levels of TCR/CD3 expression.

Based on our observations of thymocyte development and previous reports (Alarcon *et al.*, 1988; Sussman *et al.*, 1988; Sancho *et al.*, 1989; Weissman *et al.*, 1989; Wileman *et al.*, 1990; Exley *et al.*, 1991; Hall *et al.*, 1991), the ζ homodimer is required for assembly and surface expression of the TCR/CD3 complex. Our surprise observation that intestinal IELs of $\zeta^{-/-}$ mice expressed normal levels of TCR/CD3 complexes implied that another similar protein had rescued the function of the ζ protein during assembly of the TCR/CD3 complex. The premiere candidate for this was the FcεRIγ chain, which is expressed on basophils, mast cells, macrophages and NK cells, and which shares structural and functional homology to the ζ chain (Ra *et al.*, 1989a,b; Reth, 1989; Benhamou *et al.*, 1990; Lanier *et al.*, 1991; Ravetch and Kinet, 1991; Rodewald *et al.*, 1991; Romeo and Seed, 1991).

To assess whether the FcεRIγ chain was indeed associated with the TCR/CD3 complex in the si-IELs of $\zeta^{-/-}$ mice, cell lysates were first immunoprecipitated with an anti-CD3-ε antibody and then analyzed by Western blot analysis using an antiserum against the FcεRIγ chain (Orloff *et al.*, 1990). A protein with the apparent molecular weight of a FcεRIγ chain homodimer (~16 kDa) was detected in the IEL lysates from both $\zeta^{-/-}$ and $\zeta^{+/-}$ mice (Figure 7). In addition, a protein with the apparent molecular weight of a ζ−FcεRIγ heterodimer (~24 kDa) was detected in the IEL lysates of control mice but not in the IEL lysates from $\zeta^{-/-}$ mice. γ−γ homodimers and γ−ζ heterodimers were not present in splenic T cell lysates from either mouse strain. The presence of ζ−ζ homodimers was detected in control intestinal IELs but not in those from $\zeta^{-/-}$ mice (Figure 1D). The presence of a lower amount of FcεRIγ chain homodimers in $\zeta^{-/-}$ mice might be due to a lower percentage of $CD3^+$ cells or $TCR\gamma\delta^+$ cells relative to $\zeta^{+/-}$ mice (Figure 6B). These results indicate that in addition to cells expressing ζ−ζ homodimers, a normal population of mouse IELs expressed TCR/CD3 complexes containing either a FcεRIγ homodimer or a ζ−FcεRIγ heterodimer, the latter of which was absent in $\zeta^{-/-}$ mice.

Discussion

We have demonstrated that a mutation in the murine CD3-ζ gene and its alternatively spliced form, CD3-η, has a pronounced effect on normal maturation of thymocytes and development of the thymic medulla. In the absence of CD3-ζ, $CD4^-/CD8^-$ DN thymocytes progress to $CD4^+/CD8^+$ DP cells, but fail to display fully TCR/CD3 on their cell surface. Consequently, the thymus contains a higher percentage of $CD4^-/CD8^-$ DN cells, a lower percentage of $CD4^+/CD8^+$ DP and $CD4^+$ and $CD8^+$ SP cells, and an increase in the proportion of cells expressing IL2Rα and high levels of HSAg. These results lead to the concept that an incomplete (i.e. CD3-ζ^-) TCR/CD3 complex cannot support normal T cell differentiation. Signal transduction through CD3-ζ must therefore play an important, yet not indispensable, role in thymic development.

In the thymus of a wild type mouse, $CD4^-/CD8^-$ DN cells first differentiate to $CD4^+/CD8^+$ DP, $TCR/CD3^-$ cells. They progress through $TCR/CD3^{lo}$ and $TCR/CD3^{hi}$, $CD4^+/CD8^+$ DP intermediates, and become $TCR/CD3^{hi}$/$CD4^+$ or $CD8^+$ SP mature cells after undergoing thymic selection (Marrack and Kappler, 1987; Petrie *et al.*, 1990; von Boehmer, 1990). Recent studies suggest that productive rearrangement of the TCRβ gene is a prerequisite for

the progression of thymocytes from $CD4^-/CD8^-$ DN to $CD4^+/CD8^+$ DP stages. In mice lacking TCRβ gene rearrangement, such as SCID, TCR$\beta^{-/-}$, RAG-$1^{-/-}$ and RAG-$2^{-/-}$ mutant mice, thymocyte development stops at the $CD4^-/CD8^-$ DN stage (Bosma and Carrol, 1991; Mombaerts *et al.*, 1992a,b; Shinkai *et al.*, 1992), whereas in TCR$\alpha^{-/-}$ deficient mice, their development arrests at the $CD4^+/CD8^+$ DP stage (Mombaerts *et al.*, 1992b; Philpott *et al.*, 1992). In the thymus of $\zeta^{-/-}$ mice, even though the differentiation of $CD4^-/CD8^-$ DN cells to $CD4^+/CD8^+$ DP TCR/$CD3^{hi}$ cells was defective, $CD4^-/CD8^-$ DN cells could progress to TCR/$CD3^{very\ low}$ $CD4^+/CD8^+$ DP cells. Together, these results support the hypothesis that proper TCRβ gene expression is important for the progression of $CD4^-/CD8^-$ DN cells to $CD4^+/CD8^+$ DP cells and is independent of the need for CD3-ζ. This is consistent with the observation that TCRβ transgenic but not TCRα transgenic/RAG-2 deficient mice show the development of $CD4^+/CD8^+$ DP cells which express low levels of TCR/CD3 complexes that lack ζ chains (Shinkai *et al.*, 1993). During the transition of $CD4^-/CD8^-$ DN cells to $CD4^+/CD8^+$ DP, TCR/$CD3^{very\ low}$ stages, the CD3-ϵ and perhaps the CD3-γ and -δ chains may play a major role in transducing signals. By contrast, the ζ chain appears to participate in later thymocyte differentiation processes leading to TCR/$CD3^{hi}$ expression. Its role in signal transduction and receptor assembly may therefore be temporally regulated during this transition and perhaps include facilitating the assembly of the TCRα chain in TCR complexes (Shinkai *et al.*, 1993).

Interestingly, $CD4^-/CD8^-$ DN and $CD4^+/CD8^+$ DP thymocytes, as well as splenocytes, from ζ mutant mice show no defect in their ability to rearrange productively Vβ4 to Jβ2.2 and to Jβ2.5 gene segments. The $CD4^-CD8^-$ population is composed of a heterogeneous population of cells, which contains at least four distinct phenotypically defined subpopulations. A model has been proposed in which these subpopulations would be, temporally, $CD44^+/CD25^-/HSA^-$, $CD44^+/CD25^+/HSA^+$, $CD44^-/CD25^+/HSA^+$ and $CD44^-/CD25^-/HSA^+$ (Godfrey *et al.*, 1993). It is suspected that TCRβ chain gene rearrangement is not widespread at the earliest of these stages, but it may be starting. By the third stage ($CD44^-/CD25^+/HSA^+$), β chain gene rearrangement is complete. If some requirement for a productive rearrangement is then imposed, cell loss will occur appreciably before the transition to the DP state. If the fourth population above ($CD44^-/CD25^-/HSA^+$) is the major subgroup in the DN cell pool and has survived due to the presence of a productive TCRβ chain gene, it will have a skewed β chain gene rearrangement from a stochastic distribution to that which is seen here in this report. Consistent with this hypothesis is the observation in TCR$\beta^{-/-}$ mice that there is a blockade preventing the transition from the third stage to the fourth (Mombaerts *et al.*, 1992b).

Despite severely defective thymocyte differentiation in $\zeta^{-/-}$ mice, their spleen and lymph nodes contained a relatively normal number and ratio of $CD4^+$ and $CD8^+$ SP cells. The ratio of $CD4^+$ and $CD8^+$ peripheral T cells is determined in part by negative and positive selection processes in the thymus (for a review see Marrack and Kappler, 1987; von Boehmer, 1990). For instance, in mice lacking major histocompatibility complex (MHC) class I antigens, virtually no $CD8^+$ T cells were detected in the spleen and lymph nodes (Koller *et al.*, 1990; Zijlstra *et al.*, 1990). Similarly, mutant mice lacking MHC class II antigens show nearly complete elimination of $CD4^+$ T lymphocytes from the peripheral lymphoid organs (Cosgrove *et al.*, 1991; Grusby *et al.*, 1991). Thus, the observation that a normal ratio of $CD4^+$ to $CD8^+$ SP cells was detected in the spleens and lymph nodes of all $\zeta^{-/-}$ mutant mice tested inevitably suggests that thymic selection had taken place. This selection can presumably operate independently of conventional TCR upregulation.

The pathway by which the presumably small outflow of thymocytes expands to form a much larger population of peripheral T cells remains unknown. In the absence of normal levels of TCR/CD3, several stimulation pathways, such as CD2, CD28, Ly6 A/E and CD44, may trigger peripheral T cell expansion. Alternatively, the proliferation of T cells may have resulted from stimulation by unknown mitogenic stimuli, such as bacterial superantigens or self-antigens present in $\zeta^{-/-}$ mice. Whichever mechanism leads to the expansion of splenic T cells in $\zeta^{-/-}$ mice, it should also account for their lack of IL2R expression due to the fact that IL2R is normally upregulated upon activation of conventional T cells. Alternatively, peripheral T cells in $\zeta^{-/-}$ mice might accumulate to a relatively normal level due to a prolonged lifespan, in which case the steady state levels may be determined by homeostatic control of T cells.

The ζ chain can be expressed in the absence of TCR/CD3 in human NK cells and associate with CD16 and FcϵRIγ chain, although such associations have not been demonstrated in the mouse (Anderson *et al.*, 1989, 1990; Lanier *et al.*, 1989). Our results showed that $\zeta^{-/-}$ mice still retained normal, if not higher, levels of NK cell activity in cytotoxic assays. Therefore, the ζ chain is not essential to mouse NK cell function and the potential role of the ζ chain in murine NK cells *in vivo* awaits further analysis.

The most striking discovery of the current study is that intestinal IELs of $\zeta^{-/-}$ mice contain the only lymphocyte population that can express normal levels of TCR/CD3. The fact that not all $Thy1^+$ IELs express TCR/CD3 indicates that the requirement for ζ chain varies among different T cells. Certain T cells have a strict requirement for ζ chain, whereas others can either do without ζ, or substitute for it with different molecules, such as the FcϵRIγ chain. The FcϵRIγ chain is structurally and functionally homologous to the ζ chain (Reth, 1989; Benhamou *et al.*, 1990; Orloff *et al.*, 1990; Ravetch and Kinet, 1991; Romeo and Seed, 1991). Our results demonstrate that while FcϵRIγ homodimers do associate with the TCR/CD3 complexes found on intestinal IELs, they are absent from splenic T cells. Furthermore, a $\zeta-$FcϵRIγ heterodimer cannot be detected in mutant IELs, although they are clearly present in the control cells. It remains to be determined whether all normal intestinal IELs express a combination of these dimers, or whether IELs expressing exclusively $\zeta-\zeta$, $\zeta-\gamma$ or $\gamma-\gamma$ dimers exist. The influence of the FcϵRIγ chains on normal T cell development in the intestine needs further analysis.

The predominance of TCR/$CD3^+$ T cells among the intestinal IELs of $\zeta^{-/-}$ mice suggests that they are a unique cell lineage that derives extrathymically. Several studies have shown that the intestine possesses functions similar to the thymus which allow the differentiation of T cells from pre-thymic stem cells (Rocha *et al.*, 1991, 1992; Mosley and

Klein, 1992; Poussier *et al.*, 1992). As examples, intestinal IELs, such as the CD8αα$^+$ cells, probably derive extrathymically under control of intestinal epithelial cells; moreover, engraftment of fetal intestine into the kidney capsule of athymic mice can support the development of T cells derived from bone marrow stem cells. Extrathymic differentiation of T cells may also occur in aged nude mice. However, the mechanisms underlying the differentiation of the presumably extrathymically derived TCR/CD3$^+$ intestine IELs in ζ$^{-/-}$ mice are probably different from those of TCR/CD3-expressing cells in nude mice. In the latter, lymphocytes expressing the TCR/CD3 molecules are not restricted to the intestine but also populate the spleen, lymph nodes, skin and liver (Rocha *et al.*, 1992).

Alternatively, the unique IEL population in ζ mutant mice may arise from hematopoietic stem cells that mature in the thymus but are later induced to assemble the FcεRIγ into their TCR/CD3 complex. Evidence that such a replacement can occur was reported recently by Mizoguchi *et al.* (1992). In their study, splenic T cells from mice which had been injected with a colon tumor cell line expressed TCR/CD3 complexes that contained the FcεRIγ in place of the ζ chain. Perhaps intestinal IELs of ζ mutant mice alter the original composition of their TCR/CD3 complexes in response to signals in the intestinal microenvironment.

Previous reports showed that intestinal IELs respond very poorly to T cell stimuli compared with splenic T cells (Mowat *et al.*, 1986, 1989; Mosley *et al.*, 1991; Barrett *et al.*, 1992; Gramzinski *et al.*, 1993), yet these cells still possess normal cytotoxic responses (Lefrançois and Goodman, 1989; Viney *et al.*, 1990; Guy-Grand *et al.*, 1991). Our studies could not demonstrate a proliferative response of ζ mutant IELs; however, the tumor-induced FcεRIγ chain-bearing T cells, referred to above, maintain their proliferative responses to T cell stimuli and secrete lymphokines, but have blunted TCR/CD3-dependent Ca^{2+} flux response and abrogated cytotoxicity against the tumor (Mizoguchi *et al.*, 1992). Possibly, separate sets of IELs with different TCR compositions may provide the intestine with a panoply of responses to the enormous variety of enterotropic antigens encountered. A reduced proliferative capacity but normal cytolytic function might avoid harmful inflammatory reactions, e.g. those leading to inflammatory bowel disease.

Materials and methods

Vector construction

Genomic clones for CD3-ζ were isolated from a strain 129/Sv phage genomic DNA library derived from D3 ES cells (Doetschman *et al.*, 1985). A total of 9.8 kb of sequence was cloned as three contiguous *Bam*HI fragments (4.2, 3.3 and 2.3 kb) using ζ cDNA fragments as probes. *Bam*HI sites divide the first two fragments within exon 2 and the second two fragments within exon 4. The 4.2 kb fragment contains the 3′-end of the first intron and the first 23 bp of exon 2. The 2.3 kb *Bam*HI fragment contains the remaining 81 bp of exon 2 and continues through to the *Bam*HI site internal to exon 4. The 3.3 kb fragment was used to isolate a diagnostic flanking probe from a region 3′ of the expected site of recombination.

Vector construction was carried out in a version of pUC18 which contains a modified polylinker. First, a synthetic linker containing stop codons in all three reading frames was placed between the 3′-end of the 4.2 kb *Bam*HI fragment (adjacent to the internal portion of exon 2) and the 5′-end of a neomycin resistance expression cassette (PGKneo; Tybulewicz *et al.*, 1991) which lacked a polyadenylation sequence. An expression cassette for the HSV thymidine kinase gene, MC1TK, was placed 5′ of the 4.2 kb *Bam*HI fragment to allow for selection against random insertion events using FIAU [1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 iodouracil; Bristol Myers]. Finally, a polyadenylation site from pSV2CAT was isolated and inserted just upstream of the 2.3 kb *Bam*HI fragment to yield the construct shown in Figure 1. We took this approach due to difficulties in directly cloning a polyadenylated version of PGKneo adjacent to the 2.3 kb *Bam*HI fragment. The targeting vector was separated from plasmid sequences by digestion with *Not*I prior to electroporation.

Transfection and screening of CD3-ζ mutant ES cell clones

Methods for culturing ES cells, DNA electroporation, isolation of ES cell clones and extraction of cellular DNA are as described (Chen *et al.*, 1993). Approximately 1 × 10^7 AB-1 ES cells were transfected with 20 μg of the targeting vector and grown under selection using G418 and FIAU. Targeted clones were identified by Southern blot analysis of ES cell DNA digested with *Xba*I, electrophoresed in 0.9% agarose gels and transferred to Genescreen nylon filters. The filters were hybridized with a 300 bp flanking probe derived from the 5′-end of the 3.3 kb *Bam*HI fragment that is contiguous with the 2.3 kb *Bam*HI fragment. DNA probes were prepared by random hexamer labeling with [α-^{32}P]dCTP.

Generation of CD3-ζ$^{-/-}$ mice

AB1 embryonic stem cells containing one targeted allele were injected into C57BL/6 blastocysts followed by transfer to pseudopregnant foster females *in utero*. Male agouti chimeras were bred to C57BL/6 females and germline transmission was ascertained by the presence of agouti coat color and verified by Southern blot analysis of tail genomic DNA as described above. Siblings heterozygous for the mutation were crossed to derive homozygous offspring. Mice at 8–12 weeks of age were used for studies unless otherwise stated. The results of normal control mice shown in the report were obtained from ζ$^{+/-}$ heterozygous mice. However, we did not find any significant differences between the ζ$^{+/-}$ and ζ$^{+/+}$ mice in these studies.

Isolation of intestinal IELs

Dissected small and large intestines were washed with HBSS, cut into 5 mm pieces and incubated with shaking at 37°C in RPMI 1640 medium (2% FCS, L-Glu, antibiotics) for 30 min. The incubated intestine fragments were then vigorously shaken in the same medium for 15 s three times. This incubation and shaking process was repeated once. The resulting cells were resuspended in 44% Percoll solution which was then layered on top of a 67.5% Percoll solution and centrifuged at 600 *g* for 20 min at 20°C. Cells at the interface were collected and washed before being further processed. The cell preparation contained 1–10% B220$^+$ cells as shown by flow cytometry.

Flow cytometry analysis

Cells were resuspended in PBS/0.5% BSA/0.05% NaN_3 buffer and incubated at room temperature with hamster, mouse and rat serum (1:10 dilution of each serum) for >10 min before adding primary antibodies. The treated cells were then incubated with specific monoclonal antibodies at 4°C for 40 min and washed once with the buffer and fixed with 1% formaldehyde. Stained cells were analyzed using a FACStar Plus (Becton-Dickinson). Isotypic antibodies were used for negative control staining. Forward and right-angle light scatter were used to exclude dead cells. Antibodies included: FITC-labeled Thy1.2 (53-2.1), anti-CD3-ε (2C11 and 500A2), anti-TCRαβ (H57), -γδ (GL3) and Vγ3 (536), CD8α (Ly2, 563-6.7), CD8β (Ly3, 53-5.8), Ly6A/E (D7), CD44 (Pgp-1, IM7), IL2R (CD25, 7D4) and HSAg (J11d); PE-labeled Thy1.2, CD4 (RM4-4), CD8α and B220 (DNL-1.9); biotinylated anti-Thy1.2, anti-B220, anti-CD3 (2C11 and 500A2), anti-TCRαβ and anti-TCRγδ (all from Pharmingen, San Diego, CA). The splenocytes used in some of the flow cytometry staining were T cells purified using a T cell affinity column as described under proliferation assay.

PCR analysis

Using 500 ng genomic DNA as substrate, PCRs were performed under standard conditions (30 cycles) in a Perkin Elmer thermocycler. Products were separated on agarose gels and bands were purified as described (Maniatis *et al.*, 1982). The primers used included: Vβ4, GAAGCCTCT-AGAGTTCATGT; Jβ2.2, TACTTTGGTGAAGGCTCAAAGCTG; Jβ2.5, TACTTTGGGCCAGGCACTCGGCTC.

Population analysis

PCR products were digested with enzymes *Hin*fI and *Alu*I for Vβ4 to Jβ2.2 joining, *Hin*fI and *Rsa*I for Vβ4 to Jβ2.5 joining. Fragments were labeled either with Klenow using one radioactive and three non-radioactive dNTPs for complete filling-in, or by the exchange reaction of T4 polynucleotide kinase using [γ-^{32}P]ATP as described in Maniatis *et al.* (1982). Fragments were separated on an 8% denaturing polyacrylamide–urea gel which was dried for autoradiography. Sequencing ladders were calibrated by reference

to published sequences and by direct comparison with labeled restriction fragments of known length.

Proliferation assay

Splenic T cells (purified by using T cell affinity column, Biotex Laboratory Inc., Edmonton, Alberta) and intestinal IELs isolated after Percoll density gradient were incubated (5×10^4/well) with anti-CD3 (2C11, 1 μg/well), ConA (5 μg/ml) and ionomycin (250 ng/ml) in the presence of T cell-depleted splenocytes (5×10^5/well) plus PMA (2 ng/ml) in flat-bottomed 96-well plates at 37°C for 72 h. The cells were pulsed with 1 μCi [^{3}H]thymidine/well (New England Nuclear, MA) for the last 18 h and were harvested using a PhD harvester.

NK activity and ADCC assay

NK cell-mediated cytotoxic lysis against YAC-1 target cells was determined by a standard 4 h (or 20 h) ^{51}Cr release assay as described (Biron *et al.*, 1987).

ADCC was performed as described (Rodewald *et al.*, 1992) with the modification that 3 mM 2,4,6-trinitrobenzene sulfonic acid (TNP) was used instead of 30 mM. The anti-TNP antibody was a gift from Dr P.Moingeon.

Western blot analysis

The analyses were performed as described with modifications (Sancho *et al.*, 1992). Briefly, cells (2×10^7) were lysed in 1 ml of 1% Surfact-Amps^{TM}35 (Pierce, Rockford, IL) lysis buffer (150 mM NaCl, 0.14% triethanolamine, 1 mM EDTA, 10.8 mM iodoacetamide, 1 mM PMSF and 1 mg of leupeptin, pepstatin and antipain). Immunoprecipitation was done using purified anti-CD3ε antibody. Proteins were separated by electrophoresis on 12.5 or 15% SDS–polyacrylamide gels, followed by transfer to PVDF membranes (Immobilon, Millipore Corp.). Membranes were blocked by incubation fo 16 h in 5% non-fat milk buffer and then reacted with anti-ζ monoclonal antibody (1C10.1) recognizing RRRGKGHDGLYQGLSTAT-KDTYD-OH peptide or anti-FcεRIγ chain antiserum (Orloff *et al.*, 1990).

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