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CD3δ deficiency arrests development of the αβ but not the γδ T cell lineage

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The CD3 complex found associated with the T cell receptor (TCR) is essential for signal transduction following TCR engagement. During T cell development, TCR-mediated signalling promotes the transition from one developmental stage to the next and controls whether a thymocyte undergoes positive or negative selection. The roles of particular CD3 components in these events remain unclear. Indeed, it is unknown whether they have specialized or overlapping roles. However, the multiplicity of CD3 components and their evolutionary conservation suggest that they serve distinct functions. Here the developmental requirement for the CD3δ chain is analyzed by generating a mouse line specifically lacking this component (δ–/– mice). Strikingly, CD3δ is shown to be differentially required during development. In particular, CD3δ is not needed for steps in development mediated by pre-TCR or γδTCR, but is required for further development of thymocytes expressing αβTCR. Absence of CD3δ specifically blocks the thymic selection processes that mediate the transition from the double-positive to single-positive stages of development.

Keywords: CD3 complex/gene targeting/T cell development

Introduction

The T cell receptor (TCR) complex consists of the TCR heterodimer associated with the CD3 chains: δ, ε, γ and ζ. The TCR heterodimer is comprised of either α/β or γ/δ subunits, which are expressed in a mutually exclusive manner on distinct T cell lineages, i.e. αβ and γδ T cells. The variable and clonotypic TCR heterodimers encode antigen specificity, while the invariant CD3 components possess both structural and signalling roles.

The basic structural role of CD3 components is demonstrated by the fact that TCR heterodimers do not reach the cell surface in the absence of a minimal CD3 complex. Thus the lack of CD3ε or ζ invariably leads to the absence of TCR surface expression (Sussman *et al.*, 1988; Hall *et al.*, 1991; Kappes and Tonegawa, 1991). CD3δ and γ, on the other hand, can be structurally dispensable, since transfected cells expressing all TCR/CD3 components, except CD3δ or γ and PBLs of CD3γ-deficient patients, still express substantial surface TCR (Kappes and Tonegawa, 1991; Perez-Aciego *et al.*, 1991). Since other cases have been described where surface TCR expression does depend on CD3δ and γ (Buferne *et al.*, 1992; Geisler, 1992), the rules governing TCR export are apparently somewhat variable with respect to these two components. The overall stoichiometry of the TCR complex has not yet been established precisely. CD3ε and ζ occur in at least two copies per TCR complex (Baniyash *et al.*, 1989; de la Hera *et al.*, 1991), while CD3δ and γ are thought to be present in only one copy each. ε subunits form heterodimers with δ and γ (Alarcon *et al.*, 1988; Koning *et al.*, 1990; Manolios *et al.*, 1990), which themselves pair preferentially with TCRα and β respectively (Brenner *et al.*, 1985; Koning *et al.*, 1990; Manolios *et al.*, 1990). On the basis of these observations the overall stoichiometry of the TCR complex is commonly given as TCRαβ/CD3γδε$_2$ζ$_2$.

The signalling function of CD3 components is demonstrated by the fact that antibody crosslinking of chimeric proteins in which individual CD3 cytoplasmic domains have been fused to unrelated surface proteins is sufficient to mediate T cell activation (Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Romeo and Seed, 1991; Eshhar *et al.*, 1992; Romeo *et al.*, 1992a,b). CD3 chains possess one or more copies of a conserved signalling motif, the immunoreceptor tyrosine-based activation motif (ITAM), in their cytoplasmic domains (Reth, 1989; Cambier, 1995). Due to significant sequence variation between cytoplasmic domains, including at positions within ITAMs, different CD3 components exhibit preferences in their interactions with intracellular signalling factors (Ravichandram *et al.*, 1993; Exley *et al.*, 1994; Isakov *et al.*, 1995; Osman *et al.*, 1996) and induce different patterns of protein phosphorylation upon activation (Letourneur and Klausner, 1992). There is evidence for both redundant and specific signalling functions for different CD3 components. Activation of T cell hybridomas or lymphomas can be mediated by each of the CD3 signalling domains by itself, indicating a redundant function (Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Romeo and Seed, 1991; Eshhar *et al.*, 1992; Romeo *et al.*, 1992a,b). On the other hand, primary resting T cells can be activated by signalling through the complete CD3 complex but not the individual cytoplasmic domain of CD3ζ, suggesting that this function may require a specific CD3 component other than ζ (Brocker and Karjaleinen, 1995). The specific pairing of

TCRα and β with CD3δ and γ respectively may endow the TCR chains with separate signalling capabilities, which could allow different signalling responses depending on how a ligand affects the TCR's conformation. This may have some relevance to the observation that different TCR ligands can induce qualitatively different signals (Janeway, 1995).

Differential signalling through the CD3 complex is essential for both mature T cell function and for thymic education of immature T cells. Thus immature $CD4^+CD8^+$, double-positive (DP) thymocytes belonging to the αβ T lineage can undergo either apoptosis (negative selection) or further differentiation to the $CD4^+CD8^-$ and $CD4^-CD8^+$, single-positive (SP) stages (positive selection) depending on the nature of the interaction with intrathymic selecting ligands. In the case of the γδ T cell lineage, there is some evidence for positive selection, in so far as thymocytes expressing class I-specific γδ TCR transgenes fail to mature in β_2-microglobulin deficient mice (Pereira *et al.*, 1992; Wells *et al.*, 1993). The occurrence of some type of negative selection is suggested by the fact that maturation of self-reactive γδ T cells is blocked (Bonneville *et al.*, 1990; Dent *et al.*, 1990). The selection of γδ T cells differs from that of αβ T cells, in that recognition of antigen by γδTCR does not necessarily require antigen processing (Schild *et al.*, 1994) and γδ thymocytes do not usually express the CD4 and CD8 coreceptors that so clearly mark the stages of αβ thymocyte progression. The present study examines the *in vivo* requirement for the CD3δ chain in TCR surface expression and for T cell development by eliminating CD3δ from whole animals through gene targeting.

Results

Generation of CD3δ-deficient mice

Mice lacking expression of the CD3δ protein, δ–/– mice, were generated via deletion of exon 2, which encompasses most of the protein coding region (Figure 1). Northern blot and RT–PCR analysis confirmed that δ–/– thymocytes lacked any intact CD3δ mRNA (although some aberrant truncated transcripts were produced), but expressed qualitatively and quantitatively normal transcripts for the closely linked CD3 ε and γ genes (data not shown). Homozygous δ–/– mice were viable and reproduced normally when maintained under specific pathogen-free conditions.

αβ T cell development in δ–/– mice

Development of αβ T cells was examined in δ–/– mice. Total cellularity of the thymus was normal or slightly increased (Table I). Normally αβ T cells go through three distinct stages during thymic development defined by expression of the CD4 and CD8 surface markers. These are, in order of increasing maturity, $CD4^-CD8^-$, (DN), $CD4^+CD8^+$ DP, and $CD4^+CD8^-$ or $CD4^-CD8^+$ SP. The transitions between these stages represent critical functional checkpoints. The DN to DP transition is a checkpoint for productive TCRβ rearrangement and is mediated by the pre-TCR complex (Groettrup *et al.*, 1993). Pre-TCR contains TCRβ in association with the surrogate pTα chain (Saint-Ruf *et al.*, 1994), but lacks TCRα. Interestingly, δ deficiency had no effect on this transition, as DP thymocytes were generated in normal numbers (Figure 2 and Table I). The second transition, from DP to SP, is a checkpoint for selection of useful TCR specificities and deletion of self-reactive TCR specificities (positive and negative selection) and is mediated by the αβTCR complex. This step was severely impeded in δ–/– mice, as both the generation of SP thymocytes, as well as the stepwise upregulation of surface αβTCR expression normally observed on DP thymocytes undergoing positive selection (Guidos *et al.*, 1990), were essentially abolished (Figure 2). Surface TCR expression on δ–/– thymocytes was similar to that on TCR^{low} DP thymocytes from normal mice. Positive and negative selection of αβ thymocytes were further tested by introducing the model αβTCR transgenes HY and AND (Kisielow *et al.*, 1988; Kaye *et al.*, 1989) into the δ–/– background. No enhancement of positive selection was observed for either transgenes (Figure 3). Negative selection was equally impeded in δ–/– mice, as DP thymocytes expressing the HY transgenes were not deleted in a male background, in which the specific antigen is expressed (Figure 3).

TCR assembly in δ–/– thymocytes

Radioiodination studies confirmed that δ–/– thymocytes expressed low levels of TCR complex at the cell surface containing CD3 ε, γ and ζ in addition to disulfide-linked TCR chains (Figure 4A). To ascertain whether low TCR surface expression was determined by a block in assembly, intracellular formation of the TCR complex was examined. Total δ–/– thymocytes were metabolically labelled and immunoprecipitated with anti-CD3ε antibody. αβTCR heterodimers were readily detected in association with CD3γ and ε (Figure 4B). Intracellular TCR complexes from δ–/– thymocytes contained relatively little CD3ζ compared with δ+/+ controls (Figure 4B), which is consistent with the blockade of development at the TCR^{low} DP stage. Inefficient association of ζ with the rest of the TCR/CD3 complex is seen in DP TCR^{low} cells from wild-type animals (Kearse *et al.*, 1994), and may be the primary mechanism for determining low TCR surface expression at this developmental stage (total δ+/+ thymocytes show greater coprecipitation of ζ with the rest of the TCR complex than δ–/– thymocytes, because the former contains a large fraction of DP TCR^{high} and SP TCR^{high} cells in which a much higher proportion of TCR complex is associated with ζ).

Development of γδ T cells in δ–/– mice

In contrast to αβ T cells, development of the alternate γδ T cell lineage was found to be normal in δ–/– mice. γδ T cells were found in all the usual sites, including thymus, spleen and intestine, and expressed virtually normal levels of surface TCR (Figure 5A and data not shown). Metabolic labelling studies on γδ thymocytes demonstrated the formation of an intracellular complex between the γδ TCR heterodimer and CD3 ε, γ and ζ. No novel proteins were detected that could be substituting for CD3δ in the γδ lineage (data not shown). Negative selection of γδ thymocytes in the δ–/– background was specifically tested by introduction of γδTCR transgenes, derived from the KN6 hybridoma, which encode a specificity for the non-classical MHC class I antigen $T\text{-}22^b$ (Bonneville *et al.*, 1990; Ito *et al.*, 1990). In the case of δ+/+ mice, $KN6^+$ thymocytes are positively selected in the $H\text{-}2^d$ background, which does not express

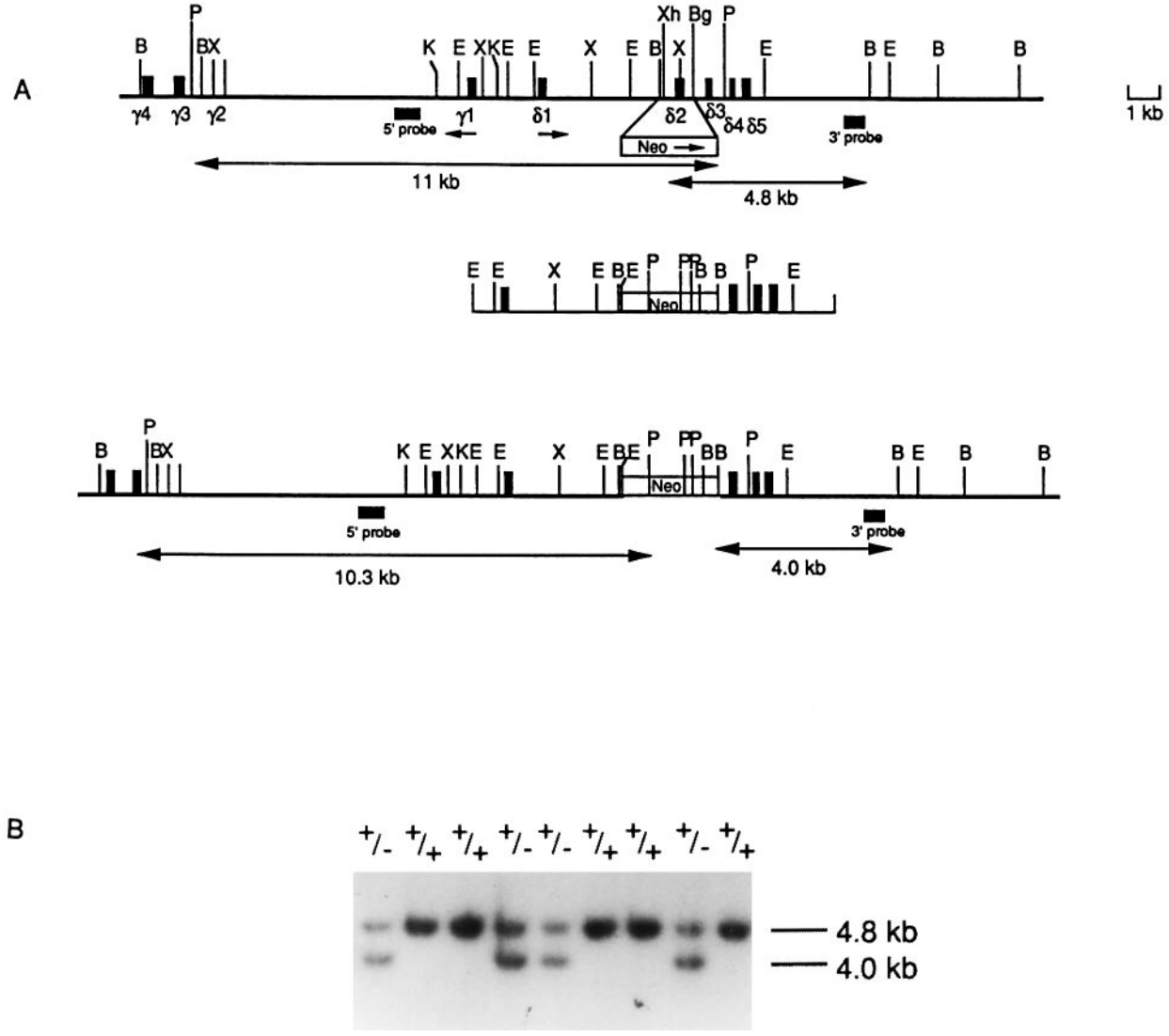

Fig. 1. Disruption of the CD3δ gene by homologous recombination. (**A**) Restriction maps of the wild-type CD3γ and δ region (top), targeting construct (middle) and CD3δ locus following homologous recombination (bottom). Restriction enzymes: B, *Bam*HI; P, *Pst*I; X, *Xba*I; E, *Eco*RI; K, *Kpn*I; Xh, *Xho*I; Bg, *Bgl*II. (**B**) Southern blot analysis of *Bam*HI digested DNA from representative ES clones hybridized with the 3′ probe. +/+ and +/– denote homozygous wild-type and heterozygous mutant cell lines respectively.

Table I. Numbers of lymphoid cells in thymus and spleen

Genotype	Total thymocyes ($\times 10^8$)	Frequency (%)				
		DP	DN	$CD4^+$	$CD8^+$	$\gamma\delta^+$
δ–/–	2.32 ± 0.46	93.8 ± 2.5	3.88 ± 0.96	<0.5	<1.0	0.84 ± 0.10
δ+/+	1.85 ± 0.25	85.4 ± 3.8	3.15 ± 1.42	8.72 ± 1.08	4.70 ± 1.55	0.32 ± 0.05
	Total splenocytes ($\times 10^8$)	Frequency (%)				
		$CD4^+$	$CD8^+$	$\gamma\delta^+$	$B220^+$	
δ–/–	1.42 ± 0.61	3.62 ± 1.35	1.42 ± 0.40	1.37 ± 0.48	67.11 ± 2.89	
δ+/+	1.55 ± 0.29	31.25 ± 4.83	10.35 ± 0.67	0.40 ± 0.06	44.76 ± 4.62	

the T-22^b antigen, but negatively selected and consequently undetectable in the periphery in the H-2^b background. The same absence of peripheral $KN6^+$ cells was observed in H-2^b δ–/– mice, indicating that negative selection of γδ thymocytes occurred normally (Figure 5B).

Peripheral αβ T cell populations in δ–/– mice

Despite the profound block in αβ T cell development in δ–/– mice a few mature αβ T cells could be found in the periphery (Figure 6 and Table I). Indeed elimination of the predominant DP subset from δ–/– thymocytes by

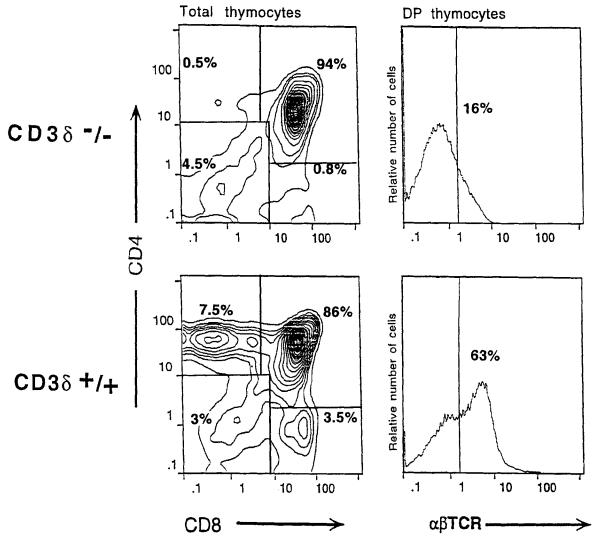

Fig. 2. Effect of CD3δ disruption on thymic development of αβ T cells. 10^5 total δ–/– and δ+/+ thymocytes from 6- to 8-week-old mice were analyzed by flow cytometry using fluorescently labelled antibodies against CD4, CD8 and TCRβ.

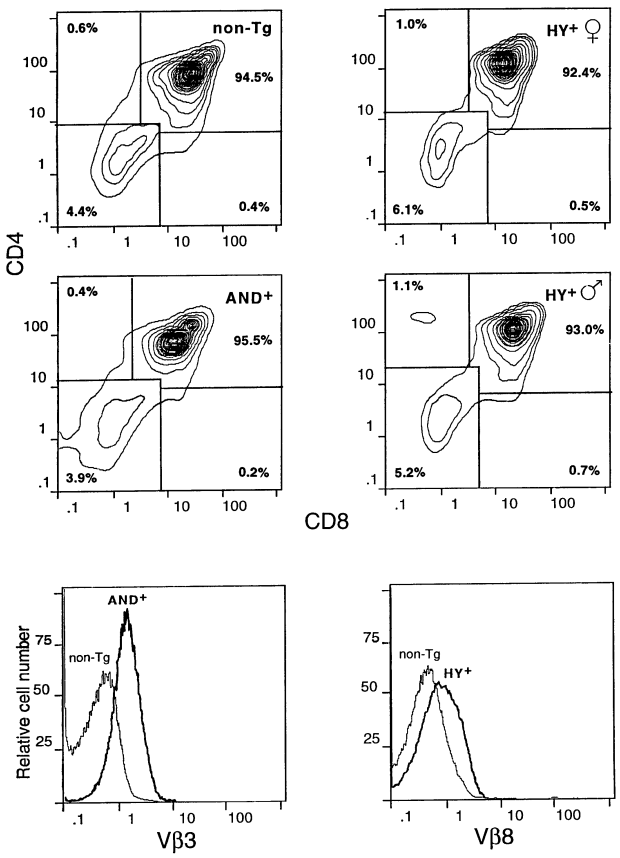

Fig. 3. Effect of AND and HY αβ TCR transgenes on thymic development in δ–/– animals. All mice were δ–/–, H-2^b and bore AND, HY or no TCR transgenes, as indicated. 10^5 total thymocytes were analyzed by flow cytometry using antibodies against CD8, CD4, TCR-Vβ3 and TCR-Vβ8 (AND and HY TCRβ chains utilize Vβ3 and Vβ8 segments respectively). Thymus cellularity was normal, i.e. $1–2\times10^8$, in all mice examined (at least four thymuses of each type were analyzed).

depletion with anti-CD8 antibody coated magnetic particles also revealed a minor population of $CD4^+$ SP thymocytes (data not shown), indicating that the developmental blockade was slightly leaky. Both δ–/– SP $CD4^+$ thymocytes and peripheral T cells expressed substantial surface TCR, albeit 5- to 10-fold reduced with respect to δ+/+ $CD4^+$ peripheral T cells (Figure 6 and data not shown). δ–/– peripheral $CD4^+$ T cells were mostly of the $CD45R^{low}$, L-$selectin^{low}$, $CD44^{high}$ memory T cell phenotype (data not shown). Metabolic labelling and immunoprecipitation analysis showed that CD3ζ associated efficiently with the TCR complex in peripheral $CD4^+$ T cells of δ–/– mice (data not shown).

Reconstitution of δ–/– mice with human CD3δ

T cell development in δ–/– mice could be rescued with a human CD3δ (hCD3δ) transgene, confirming that the defect was entirely attributable to the absence of CD3δ (Figure 7A). T cells expressing hCD3δ could be identified by their reactivity with anti-hCD3 antibodies such as MEM-57 (Figure 7A and B). Interestingly, in a subset of peripheral T cells from $hCD3\delta^+$ δ–/– mice, MEM-57 staining was undetectable, even though αβTCR surface expression remained high (Figure 7B). Down-modulation of hCD3δ surface expression was transcriptionally determined, as the level of hCD3δ transcript was reduced 7- to 10-fold with respect to β-actin mRNA in cells with undetectable surface hCD3δ (Figure 7C).

Discussion

The most striking aspect of the δ–/– phenotype is the fact that development of the αβ and γδ T cell lineages is differentially affected. This suggests that CD3δ plays a crucial signalling role in the thymic selection of αβ T cells specifically. In contrast, two previously reported knockouts of CD3 components, namely CD3ε and ζ, show equal blockades of γδ as well as αβ T cell development. Furthermore, the generation of DP from DN thymocytes is substantially or completely impaired in these mice (Liu *et al.*, 1993; Love *et al.*, 1993; Malissen *et al.*, 1993, 1995; Ohno *et al.*, 1993). Thus these blockades are both broader and of earlier onset. In interpreting the ζ–/– and ε–/– phenotypes, it is significant that TCR surface expression is entirely or almost undetectable (except for some gut intraepithelial T lymphocytes in ζ–/– mice in which FcεRIγ substitutes for CD3ζ). This demonstrates a strong *in vivo* structural requirement for ζ and ε in TCR assembly, consistent with earlier *in vitro* findings (Sussman *et al.*, 1988; Hall *et al.*, 1991; Kappes and Tonegawa, 1991). It seems probable that the developmental blockades in ζ–/– and ε–/– mice result from the fact that very low surface TCR levels are insufficient to support thymic selection. This interpretation is supported by the finding that TCR surface expression and T cell development are both at least partially restored in ζ–/– mice by the introduction of truncated ζ transgenes lacking a cytoplasmic domain, i.e. encoding structural not signalling function (Shores *et al.*, 1994; Aoe *et al.*, 1996). Interestingly, in one study although the DP to SP transition is substantially restored, the DN to DP transition remains impaired suggesting a specific role for CD3ζ-mediated signalling at this earlier transition.

It is apparent from the analysis of δ–/– mice that CD3δ

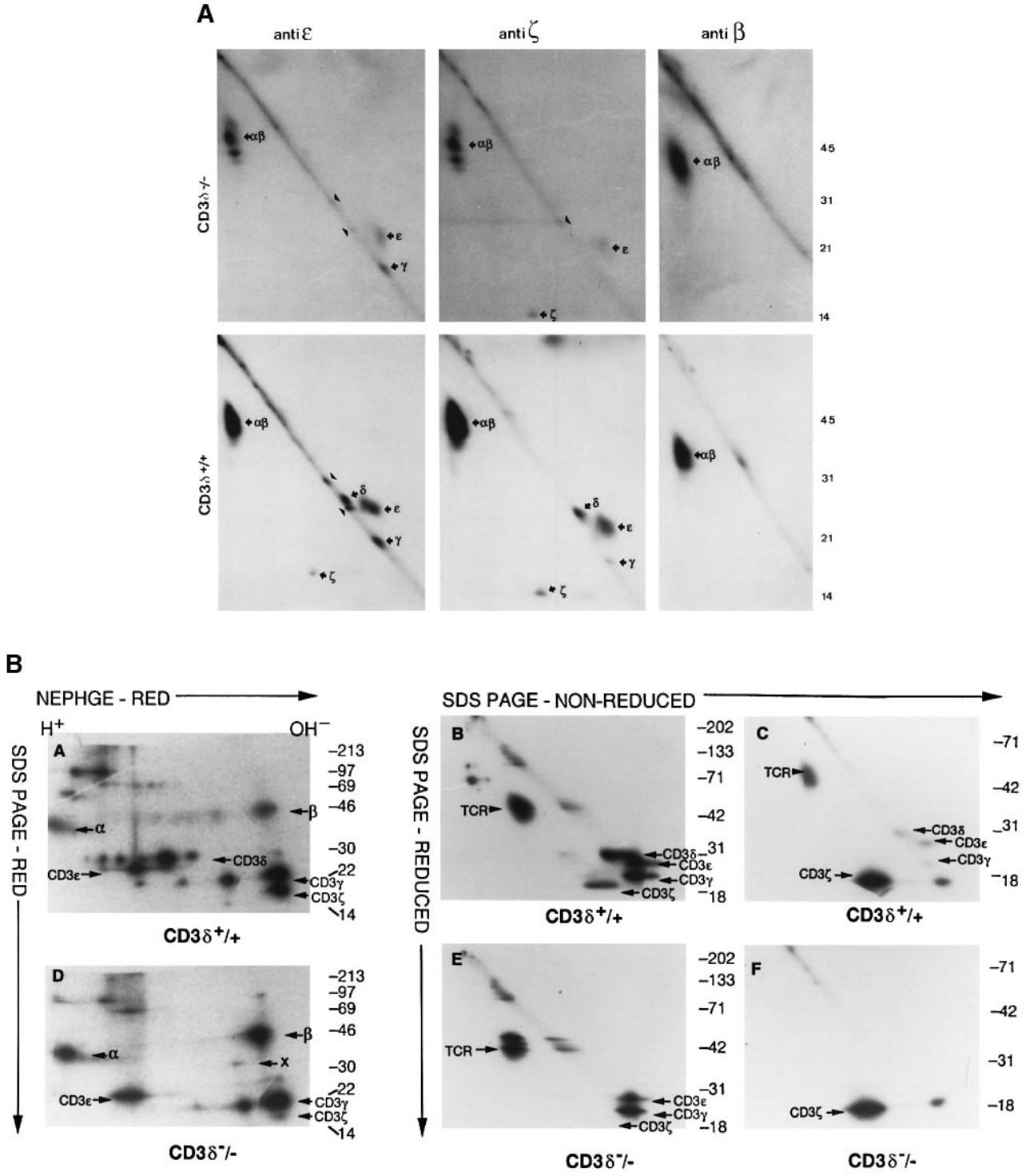

Fig. 4. Biochemical analysis of the TCR complex on δ–/– thymocytes. (**A**) 1×10^8 total thymocytes from δ–/– and δ+/+ mice were surface-radioiodinated, lysed and immunoprecipitated with the antibodies indicated. Immunoprecipitates were analyzed by 2-D non-reduced/reduced SDS–PAGE. Arrowheads show the positions of contaminating proteins. (**B**) Total thymocytes were pulse-labelled with [^{3}H]leucine, lysed and immunoprecipitated with anti-CD3ε (panels A, B, D and E) or anti-CD3ζ (panels C and F). Samples were analyzed by either 2-D non-reduced/reduced or NEPHGE/SDS–PAGE as indicated.

is not similarly structurally essential, since substantial surface TCR is observed on both αβ and γδ T cells in these animals. This is in agreement with our own previous *in vitro* study (Kappes and Tonegawa, 1991), but contradicts two studies of δ-deficient T cell variants (Bonifacino *et al.*, 1989; Buferne *et al.*, 1992). These discordant results could be reconciled by postulating the existence of different intracellular control mechanisms regulating TCR complex assembly and transport, one consequence of which would be to alternatively permit or disallow the expression of surface TCR complex lacking CD3δ. However, it should be noted that such variation is not apparent *in vivo* within peripheral δ–/– $CD4^+$cells, all of which appear TCR^+. The fact that TCR heterodimer

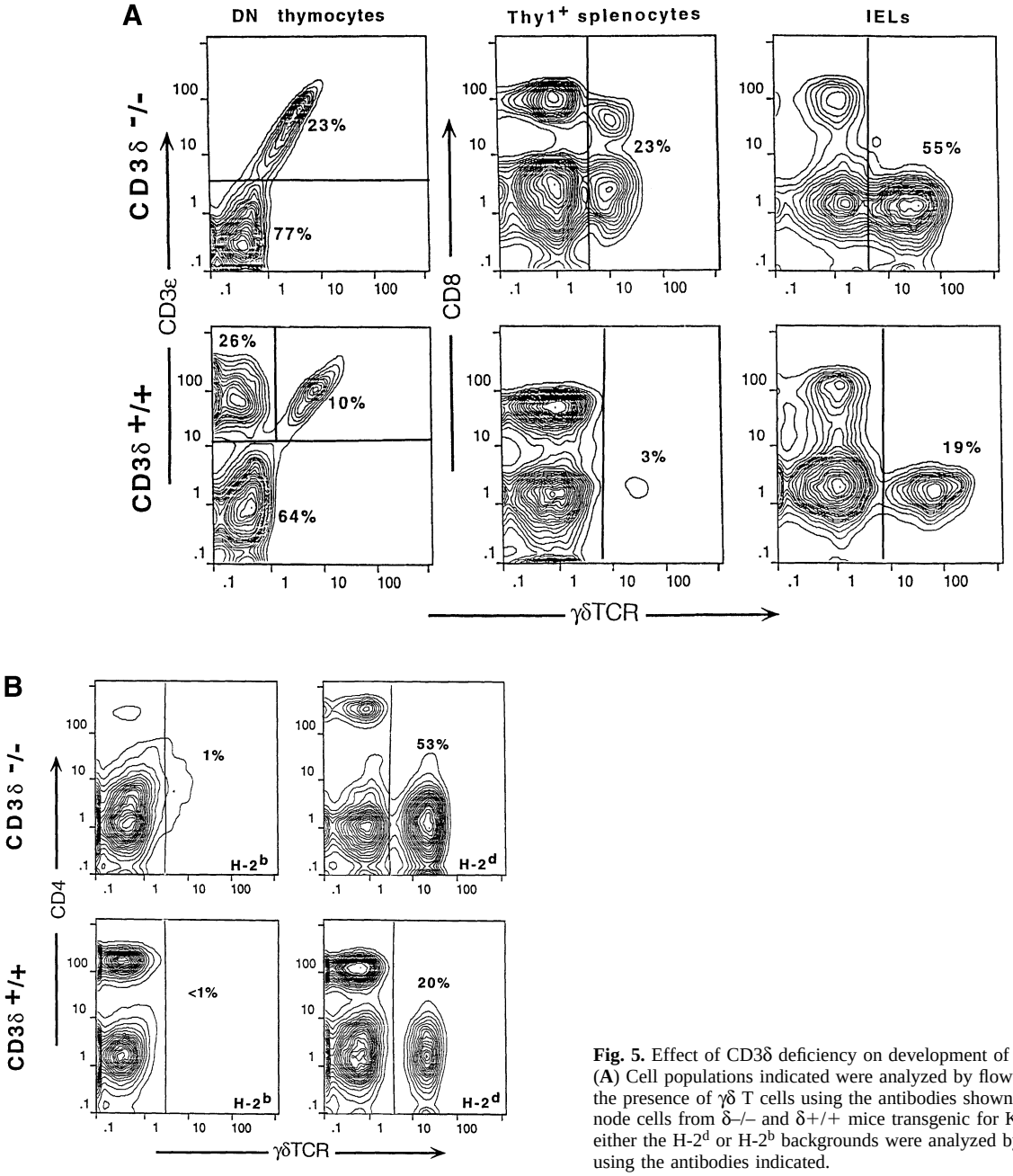

Fig. 5. Effect of CD3δ deficiency on development of γδ T cells. (**A**) Cell populations indicated were analyzed by flow cytometry for the presence of γδ T cells using the antibodies shown. (**B**) Lymph node cells from δ–/– and δ+/+ mice transgenic for KN6 γδTCR in either the H-2^d or H-2^b backgrounds were analyzed by flow cytometry using the antibodies indicated.

formation is not grossly impaired in δ–/– mice is significant for the mechanism of TCR assembly. Thus it has previously been reported that αβTCR heterodimer formation is preceded by association of TCRα monomers with CD3δ/ε subcomplexes (Kearse *et al.*, 1995), suggesting that CD3δ is required for this process. Evidently, preassociation of CD3δ with TCRα, while normally preferred, is not essential. Although TCR levels on residual mature T cells in δ–/– mice are substantial, they are reduced relative to δ+/+ mice. More significantly the level of reduction is greater for αβ than γδ T cells, i.e. 5- to 10-fold for peripheral $CD4^+$ αβ T cells but less than 2-fold for γδ T cells. This indicates some structural role for CD3δ in TCR complex formation, albeit not as significant as for ε and ζ.

Hence, two alternative hypotheses must be considered in interpreting the δ–/– phenotype; i.e. (i) that it reflects a partial reduction in TCR surface expression levels that somehow translates into alternate effects on the αβ and γδ T cell lineages, not indicative of a specific signalling function for CD3δ, or (ii) that it reflects a specific signalling function for CD3δ essential in αβ T cell development. (i) If the relative reduction of TCR levels on mature δ–/– T cells holds true for thymocytes, then αβ thymocytes would receive weaker positive selection signals than γδ thymocytes, potentially accounting for the greater impairment in their development (the degree to which αβTCR surface levels are reduced on δ–/– relative to δ+/+ DP thymocytes is hard to quantitate directly, since TCR^{low} DP thymocytes express so little surface TCR). Greater sensitivity of αβ TCR surface expression to the absence of CD3δ might be related to structural differences between γδ and αβ TCR complex or to differences in the intracellular control of TCR assembly and transport between the two lineages. Since biochemical

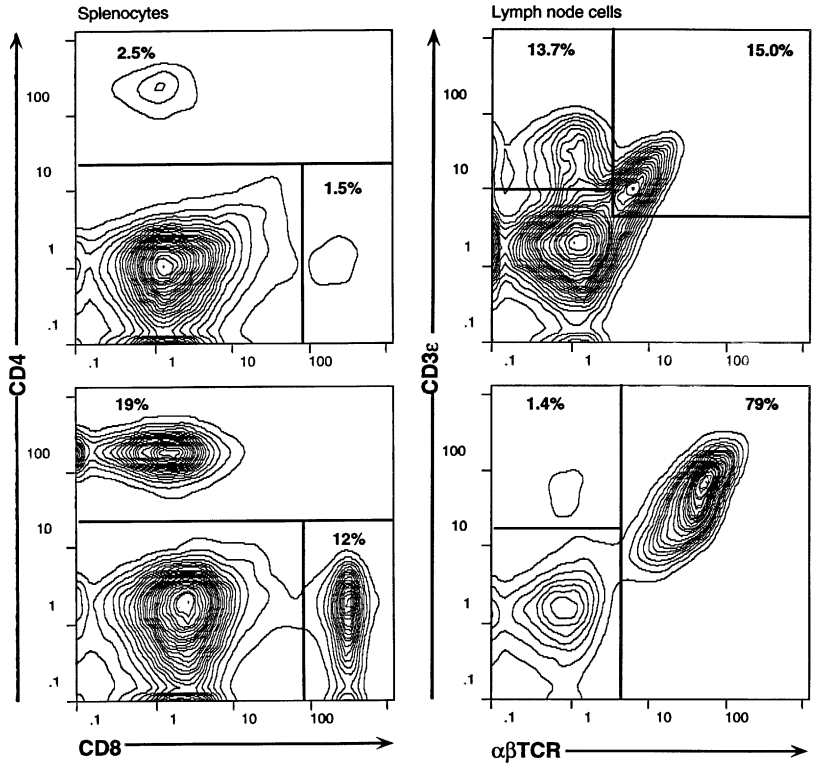

Fig. 6. Expression of surface αβTCR complex lacking CD3δ on peripheral T cells. Cell populations indicated were isolated from δ–/– mice and analyzed by flow cytometry using antibodies against CD4, CD8, TCRβ and CD3ε (TCRβ$^-$ CD3ε$^+$ populations represent γδ T cells).

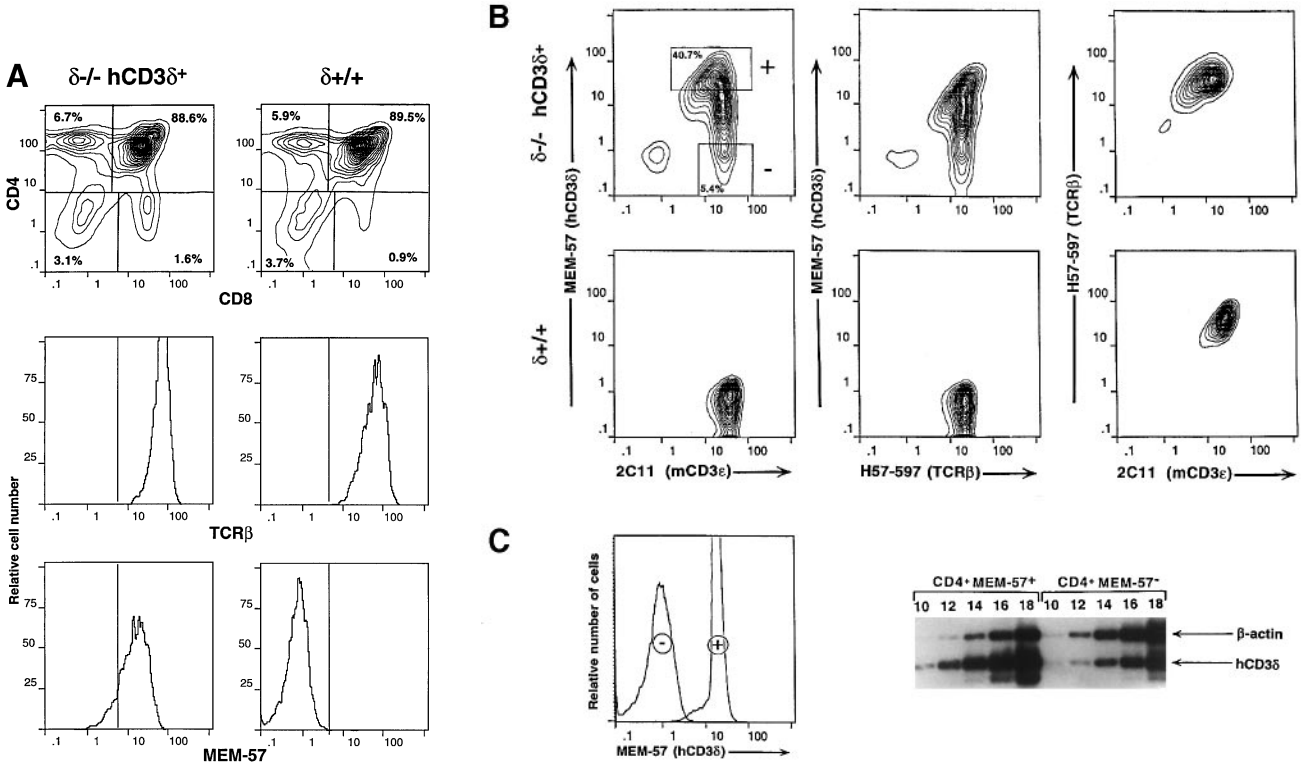

Fig. 7. Restoration of thymic development and peripheral T cell populations in δ–/– mice by a human CD3δ transgene. (**A**) 10^5 total thymocytes from a δ–/– hCD3δ$^+$ or a non-transgenic δ+/+ control were stained with fluorescently labelled antibodies against CD4, CD8, TCRβ and hCD3 (MEM-57) and analyzed by flow cytometry. (**B**) CD4$^+$ T cells from peripheral blood of δ–/– mice expressing the human CD3δ transgene or δ+/+ non-transgenic controls were stained with fluorescently labelled antibodies against TCRβ, hCD3 and mCD3ε. (**C**) Populations of 10^5 cells from δ–/– hCD3δ$^+$ animals showing high and low expression of the hCD3δ epitope by MEM-57 staining [sorting gates shown in upper left panel of (A)] were isolated by FACS. Left panel shows reanalysis after sorting. mRNA was prepared and analyzed for levels of hCD3δ transcript by RT–PCR.

comparisons of αβ and γδTCR show no differences in the CD3 complex and clearly demonstrate the presence of CD3δ in the γδTCR complex (Krangel *et al.*, 1987; van Neerven *et al.*, 1990), significant structural differences seem unlikely. It must also be pointed out that the apparent relative reduction in TCR density for $CD4^+$ cells may be deceptive. Thus virtually all δ–/– $CD4^+$ T cells belong to the subset of memory T cells, which in normal mice express 2- to 3-fold less surface TCR than other $CD4^+$ T cells (Kariv *et al.*, 1994). Hence, there may be little inherent difference in expression of αβ and γδ TCR in the absence of CD3δ.

One could still argue that a small reduction in TCR surface density has a more pronounced effect on the development of the αβ than the γδ lineage. In this respect, it is significant that the blockade in αβ T cell development occurs at the DP stage, i.e. when αβ thymocytes go through a TCR^{low} stage. Presumably DP TCR^{low} cells possess limited signalling capacity and may not tolerate any additional reduction in TCR surface density. However, it should be noted that at least some γδ T cells, namely those expressing Vγ3, also undergo a stepwise upregulation of surface TCR levels during development (Leclerq *et al.*, 1993). These Vγ3 expressing T cells develop normally in δ–/– mice and undergo the same stepwise upregulation of surface TCR (D.Kappes, unpublished data). Hence, one cannot argue that any developing T cell passing through a TCR^{low} intermediate stage is blocked in development in δ–/– mice. Evidence that the DP stage is somewhat resistant to reduced surface TCR levels is provided by mice heterozygous for a mutation at the CD3ζ locus; TCR surface expression is reduced by 2-fold in these mice without causing a noticeable impairment in the generation of SP cells (Malissen *et al.*, 1993). Conversely, while expression of HY or AND TCR transgenes in δ–/– mice increases surface TCR levels on DP thymocytes 2- to 3-fold (data not shown), there is no enhanced generation of SP cells.

In the alternative model (ii), the absence of CD3δ induces a structural alteration in the surface TCR complex, which results in a qualitative change in its signalling capacity. The partial or altered signal that results is hypothesized to be adequate for γδ but not αβ T cell development. The qualitative change in signalling could, in principle, involve any of the components of the TCR complex as they may undergo conformational changes in response to the absence of CD3δ. However, the most obvious possibility is that the missing CD3δ component itself encodes an essential function. This could involve interaction with a specific intracellular factor necessary for the initiation of a distinct signalling pathway. While no such factor is presently known, there is growing evidence that cytoplasmic tails of different CD3 components do interact differently with intracellular signalling molecules (Ravichandran *et al.*, 1993; Exley *et al.*, 1994; Isakov *et al.*, 1995; Osman *et al.*, 1996).

In a variation of this model, CD3δ might function as a docking molecule to link the TCR complex to other signalling structures; in this case the extracellular rather than the cytoplasmic domain might be critical. It may be relevant in this respect, that a null mutation of the tyrosine kinase $p56^{lck}$ also disrupts αβ T cell development, while leaving γδ T cells relatively unaffected (Molina *et al.*, 1992). There is strong evidence that direct association between the CD4/CD8 coreceptors and the TCR complex is essential for the functional interaction of $p56^{lck}$ with the TCR complex (Dianzani *et al.*, 1992; Diez-Orejas *et al.*, 1994). Furthermore, it has been reported that there is a specific association between CD4/CD8 and CD3δ (Suzuki *et al.*, 1992). Hence one possibility is that the absence of CD3δ uncouples CD4/CD8 and therefore $p56^{lck}$ from the TCR complex. Such a model would be consistent with the lack of effect of the CD3δ-deficiency on pre-TCR and γδTCR expressing cells, which normally lack coreceptors.

A further interesting question in this regard is how the few 'leaky' SP αβ T cells that mature in δ–/– mice arise. There appears to be selection for particular TCR specificities, not just random leakiness of the normal TCR repertoire, since AND TCR transgenes expressed on a δ–/– RAG-2–/– background, where endogenous rearrangements are prevented, are not positively selected at all, resulting in the complete absence of peripheral SP T cells (D.Kappes, unpublished data).

It is intriguing that CD3δ plays no role in the DN to DP transition. While CD3δ has been found to be associated with the pre-TCR complex in various cultured immature thymocyte lines (Punt *et al.*, 1991; Groettrup *et al.*, 1992; Mombaerts *et al.*, 1995), this is not the case in primary pre-T cells derived from TCRα-deficient mice, which may more accurately reflect the normal physiological situation (Jacobs *et al.*, 1994). Our data showing that CD3δ is not essential for pre-TCR-mediated maturation provide indirect support for the idea that it is not part of the pre-TCR complex *in vivo*. Since pre-TCR lacks TCRα, the coincident absence of CD3δ would reflect its known preference for association with TCRα rather than TCRβ, or conversely its limited ability to compete with CD3γ for association with TCRβ. Thus one might postulate that CD3δ is recruited to the TCR complex by TCRα after successful TCRα rearrangement, and thereby provides complete TCR complex with an essential new signalling capacity. Thus recruitment of CD3δ to the TCR complex may be crucial for letting the cell know that TCRα has rearranged successfully. Conversely, a specific requirement for CD3δ at the DP stage would provide a means of preventing the inappropriate maturation of thymocytes expressing pre-TCR.

Reconstitution of normal αβ T cell maturation in δ–/– mice by human CD3δ demonstrates that the developmental blockade observed in δ–/– mice can be entirely ascribed to the absence of CD3δ. Human CD3δ appears to substitute fully for its murine equivalent in terms of mature T cell numbers and TCR expression levels. Peripheral T cells from these reconstituted mice also provide intriguing evidence for plasticity in the composition of the surface TCR complex, since some cells express very little hCD3δ at the cell surface while supporting normal expression of other components of the TCR/CD3 complex. Down-modulation of surface hCD3δ correlates with reduced levels of hCD3δ mRNA. This down-regulation may represent a transgene-specific effect resulting from partial deletion of the transgene or position-effect variegation, as described in a number of transgenic lines (Bluethmann *et al.*, 1988; Festenstein *et al.*, 1996). Alternatively, since the hCD3δ transgene is under the control of its native albeit human transcriptional

elements, it may reflect an *in vivo* mechanism for modulating the composition and signalling capacity of the TCR complex with potential functional consequences. Previous reports have presented evidence that surface expression of different components of the TCR complex may be regulated independently, although not at the level of transcription (Kishimoto *et al.*, 1995; Ono *et al.*, 1995). In addition there is evidence for plasticity in CD3 composition of the TCR surface complex on T cells from tumour bearing mice and cancer patients (Mizoguchi *et al.*, 1992; Nakagomi *et al.*, 1993; Farace *et al.*, 1994).

Materials and methods

Generation of δ–/– mice

Cosmid clones encompassing the CD3δ and γ genes were isolated from a C57Bl/6 mouse genomic DNA library. The targeting construct was created by subcloning a 9 kp fragment containing the entire CD3δ gene and replacing a central *Xho*I–*Bgl*II fragment with a pgk–neo^r cassette. This resulted in deletion of 700 bp of genomic DNA containing CD3δ exon 2, which encodes the external domain of the CD3δ protein. The targeting construct was electroporated into C57Bl/6 ES cells (a gift of B.Ledermann and K.Burki; Ledermann and Burki, 1991) by standard methods. ES clones that had undergone homologous recombination were identified by Southern blotting using 5′ and 3′ probes located outside of the construct. Because of restriction sites derived from the pgk–neo cassette, the 11 kp *Pst*I fragment detected by the 5′ probe was reduced to 10.3 kp and the 4.8 kp *Bam*HI fragment detected by the 3′ probe to 4.0 kp. Mutant ES cells were injected into Balb/C blastocysts. Test-mating of resultant chimeras identified one male which transmitted exclusively the ES cell-derived genome. This mouse was mated to C57Bl/6 females giving rise to heterozygous mutant animals of pure C57Bl/6 background, which were then cross-bred to generate a homozygous CD3δ-deficient (δ–/–) line.

Protein analysis

For analysis of cell surface TCR complexes 10^8 total thymocytes of δ–/– or δ+/+ animals were surface-radioiodinated by the lactoperoxidase method. After iodination, cells were lysed in 1% Brij96 immunoprecipitation buffer and immunoprecipitated with anti-CD3ζ (N39), anti-TCRβ (H57) or anti-CD3ε (2C11).

For analysis of intracellular TCR complexes, 10^8 total thymocytes were labelled with [^{3}H]leucine for 90 min, chased in the presence of unlabelled leucine for 40 min and lysed in 1% digitonin buffer. Immunoprecipitations were carried out with anti-CD3ε (2C11, Pharmingen) or anti-CD3ζ (polyclonal serum #551; gift of D.Wiest and A.Singer). Immunoprecipitates were analyzed by 2-dimensional non-reduced/reduced or NEPHGE/SDS–PAGE.

Flow cytometry

10^5 cells from the thymus or peripheral lymphoid organs were incubated with the relevant combinations of fluorescently labelled antibodies and analyzed using either a Becton-Dickinson FACStar or FACscan. PE- and CyChr-labelled anti-human CD3 (MEM-57) was obtained from Caltag. FITC-, PE-, CyChr- and/or APC-conjugated antibodies against CD3ε (2C11), TCRβ (H57-597), CD4 (RM4-5), CD8 (53-6.7), CD44 (1M7), CD45RB (16A) and CD62L (MEL-14) were all purchased from Pharmingen. Populations of CD4$^+$ peripheral T cells from δ–/– hCD3δ$^+$ animals showing high and low expression of the hCD3δ epitope by MEM-57 staining were isolated by fluorescence activated cell sorting, FACS.

RT–PCR analysis

RT–PCR analysis was carried out on purified CD4+MEM-57+ and CD4+MEM-57– fractions of peripheral T cells from δ–/– hCD3δ$^+$ mice according to published protocols (Wasserman *et al.*, 1995). Briefly, mRNA was prepared from 10^5 sorted cells and aliquots were used for cDNA synthesis with MMLV reverse transcriptase. cDNA was amplified by PCR using pairs of specific hCD3δ and β-actin primers for 10–18 cycles to establish the linear range of amplification. Linearity breaks down after 14 cycles. PCR products were separated on a 1.5% agarose gel, transferred to Hybond-N$^+$ membrane (Amersham) and blotted simultaneously with radiolabelled hCD3δ and β-actin probes. Intensities of bands were quantified with a Fuji Bio-Image Analyzer. Relative levels of hCD3δ transcripts were determined by comparing hCD3δ:β-actin ratios at 12 and 14 PCR cycles between MEM-57+ and MEM-57– cell fractions. Some hCD3δ mRNA is clearly still present in the MEM-57– fraction suggesting that sorted populations are not completely pure or alternatively, that hCD3δ transcripts are severely reduced but not eliminated completely.

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