Differential Effects of ζ and η Transgenes on Early α/β T Cell Development

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Summary

The ζ-family dimers (ζ, η, and γ) are a group of structurally and functionally related proteins that are expressed in developing thymocytes and function as signal transducing subunits of the T cell antigen receptor (TCR) and certain Ig Fc receptors. ζ, η, and γ each contain one or more copies of a conserved tyrosine-based activation motif (TAM) that is known to be required for signal transduction. To examine the developmental importance of multiple or individual TAM elements we generated transgenic mice that express: (*a*) full-length (FL) ζ-chain (3 TAMs); (*b*) η-chain, a naturally occurring variant of ζ that is derived from alternative splicing (2 TAMs); or (*c*) truncated ζ-chain (CT108; 1 TAM), under the control of the human CD2 promoter and regulatory elements. Unexpectedly, we found that overexpression of the FL ζ chain caused premature termination of RAG-1 and RAG-2 expression, prevented productive rearrangement of the TCR-α and TCR-β genes and blocked entry of thymocytes into the CD4/CD8 developmental pathway. In contrast, we found that overexpression of η or CT108 had no effect on normal thymocyte maturation. These results suggest that an early signaling pathway exists in precursor TCR$^-$ thymocytes that can regulate RAG-1 and RAG-2 expression and is differentially responsive to individual members of the ζ-family dimers.

The ζ-family dimers are a group of structurally and functionally related proteins that play a central role both in receptor surface expression and signal transduction. Members of the ζ-family currently include ζ (1), its alternatively spliced form, η, (2) and the γ chain subunit of the high affinity IgE receptor, FcϵRI (3). The ζ chain was first discovered as a component of the multimeric TCR complex (4). The TCR is now known to be composed of at least seven subunits that fall into three distinct groups: (*a*) the disulphide-linked clonotypic subunits (α/β or γ/δ) which are the products of developmentally programmed gene rearrangements and confer ligand binding specificity (5); (*b*) the invariant CD3 chains, γ, δ, and ϵ, which assemble as noncovalently linked heterodimers (6); and (*c*) a disulphide-linked homo- or heterodimer composed of one or more members of the ζ-family of proteins (7).

ζ-family dimers are required for targeting expression of the TCR (8) as well as at least two other receptor complexes, FcγRIII (CD16) (9, 10) and FcϵRI (3) to the cell surface. The assembly, transport, and surface expression of these receptor complexes is dependent upon sequences that reside exclusively within the charged transmembrane domains of their respective subunits (10, 11). ζ-family dimers also function to couple the TCR (8, 12) as well as other lymphocyte cell surface molecules (Thy 1, Ly-6, CD2) (12–14) to a complex signal transduction pathway that involves mobilization of intracellular calcium stores, activation of protein kinase C, and activation of cytoplasmic protein tyrosine kinases (4, 15). Conserved sequences (termed tyrosine-based activation motifs; [TAMs][1]) within the cytoplasmic domains of the ζ, η, and FcϵRIγ chains are required for signal transduction (13, 16–19). TAM elements, which consist of a pair of tyrosines separated by 10 or 11 amino acids, are also present in the cytoplasmic domains of several other transmembrane proteins known or thought to function in signal transduction, including the CD3-γ, -δ and -ϵ chains, and the β subunit of the high affinity IgE receptor (13, 18, 20). Within this group, the ζ and η chains are unique in that they contain multiple TAMs: 3 or 2 respectively. The functional significance of the individual TAM elements or of multiple TAMs is still unknown. In T hybridoma lines, the presence of a single TAM, either in

[1] *Abbreviations used in this paper:* DP, double positive; FCM, multicolor flow cytometer; FL, full-length; SP, single positive; TAM, tyrosine-based activation motif; Tg, transgenic; TRA, Texas Red Avidin.

its native context or as part of various synthetic transmembrane chimeras, is both necessary and sufficient to mimic TCR effector functions (12, 13, 17, 18, 21). In addition, truncated ζ chain variants that are devoid of TAM elements but retain the transmembrane domain are capable of reconstituting surface expression of functional TCR complexes (as assessed by ligand binding or antibody-mediated cross-linking) (13). These observations indicate that TAMs within the CD3 chains can function as autonomous signaling modules in the absence of ζ–ζ dimers.

Although considerable insight has been gained regarding the function of ζ and related molecules in established T cell lines, their role in thymocyte development has not been specifically addressed. The TCR complex regulates thymocyte development by initiating specific signal transduction events at appropriate stages in ontogeny. TCR-mediated signaling in immature $CD4^+CD8^+$ thymocytes can result in either the deletion of self-reactive clones (negative selection) or their further maturation (positive selection) (22, 23). TCR signals also function to fix the specificity of individual thymocytes by terminating expression of the recombination activating genes, RAG-1 and RAG-2 (24). An intrinsic potential for variability in the TCR-directed signaling response derives not only from the clonotypic nature of the TCR (conferred by the α/β or γ/δ chains) but also from the ability of the TCR to assemble into distinct isoforms by association of the $\alpha\beta\gamma\delta\epsilon$ core with various permutations of ζ-family dimers, e.g., $\alpha\beta\gamma\delta\epsilon$–$\zeta\zeta$, $\alpha\beta\gamma\delta\epsilon$–$\zeta\eta$, $\alpha\beta\gamma\delta\epsilon$–$\zeta\gamma$ . . . etc. (7). Whether or not different members of the ζ-family dimers transduce quantitatively or qualitatively distinctive TCR signals that affect thymic development is presently unclear.

To address this question, we generated transgenic mice that expressed a full-length (FL) ζ chain, η chain, or a truncated ζ chain variant (CT108) that lacks the COOH-terminal half of the cytoplasmic domain (12). Thus, the transgene encoded proteins contained 3, 2, or 1 TAMs, respectively. We found that overexpression of FL ζ caused a dose-dependent arrest of thymocyte maturation characterized by incomplete rearrangement of the TCR-α and TCR-β genes, whereas overexpression of η or CT108 did not preceptibly alter the normal thymocyte developmental program. Furthermore, overexpression of FL ζ chain resulted in premature termination of RAG-1 and RAG-2 expression and upregulation of CD2 and CD5 in $CD4^-CD8^-$ TCR^- fetal thymocytes. Collectively, these results suggest that a TCR-independent signal transduction pathway functions in early thymocyte precursors and is differentially responsive to individual members of the ζ-family dimers.

Materials and Methods

DNA Constructs and Microinjection. ζ-FL and ζ-CT108 minigenes were generated by ligating a 350-bp EcoR1–Acc1 cDNA fragment (containing exons I-Va) from either plasmid pSVL-ζ or pSVL-CT108, respectively (12), with a 4.2-kb Acc1–BamH1 genomic DNA fragment encoding ζ exons Va-VIII (reference 25; Fig. 1). The η minigene was constructed using a PCR-generated EcoR1–Sal1 fragment that contained the complete η cDNA coding sequences. Transgene constructs were then made by replacing the EcoR1–BamH1 fragment in construct CD2-B (which contained human CD2 coding sequences; reference 26) with the ζ-FL, ζ-CT108, or η minigene fragments. After digestion with Sal1 and Not1, transgene DNA was separated from vector sequences by electrophoresis, extracted from agarose gel slices and resuspended in TE buffer (10 mM Tris, pH 7.4; 0.1 mM EDTA) at a final concentration of 5 ng/μl. Fertilized oocytes were obtained from inbred FVB/N mice and pronuclear injection was performed essentially as described (27).

Antibodies and Reagents. Anti-FcγRII/III mAb (2.4G2) (28) was provided by J. Titus (National Institutes of Health, [NIH]). FITC anti-CD5 mAB (52-7.3), biotin anti-CD8 mAb (53-6.72), and negative control (anti–human) mAbs (FITC-Leu-4 and biotin-Leu-4) were obtained from Becton Dickinson Immunocytometry Systems (Mountain View, CA). FITC-conjugated anti-TCRδ (GL3) mAb, anti-CD4 (RM4.5), and anti-CD2 mAb (RM2-5), as well as biotin-hamster anti–mouse CD3 (500.A2) were purchased from PharMingen (San Diego, CA). Hamster anti–mouse CD3 mAb (145-2C11) (29) and hamster anti–mouse TCR-β mAb (H57-597) (30) were purified from culture supernatant and conjugated as required in our laboratory. Texas red-conjugated streptavidin (TRA) (red fluorescence) was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Anti-ζ antibodies 528 and 551 were generously provided by A. M. Weissman (NIH). Antibody 528 was generated in a rabbit to a peptide that corresponded to amino acids 88–101 of murine ζ and recognizes both the ζ and η proteins (31); antibody 551 was generated to a peptide that corresponded to amino acids 151–164 and recognizes only ζ protein.

Multicolor Flow Cytometry (FCM). Thymocytes were stained at 4°C and washed with Hanks balanced salt solution containing 0.5% bovine serum albumin and 0.5% sodium azide. In general, cells were first incubated with anti-FcR mAb 2.4G2 to prevent nonspecific binding of conjugated mAbs. For two-color FCM studies, cells were incubated with FITC-labeled mAbs for 30–40 min, washed 3×, and then incubated with biotinylated mAbs for 30–40 min. The antibody-labeled cells were washed 3×, followed by the addition of TRA-streptavidin for 10 min. Cells were then washed 3× and analyzed by FCM. Multicolor FCM was performed using a modified Dual-Laser FACStar PLUS® or FACS II® (Becton Dickinson Immunocytometry Systems) equipped with the manufacturer's filters and photomultiplier tubes, argon ion laser for FITC excitation (488 nm), pumped dye (rhodamine 6C) laser for Texas red excitation (590 nm), and interfaced to a DEC MVAX 3500 (Digital Equipment Corporation, Marlboro, MA). All fluorescence analyses were performed by using logarithmic amplification (3-decade or 4-decade, as indicated), and data were collected and analyzed using hardware and software designed by the Division of Computer Research and Technology at the NIH. Fluorescence data were collected on 5–25 × 10^4 viable cells, as determined by electronic gating on forward light scatter and propidium iodide exclusion. Electronically-gated data were then collected in matrix form on 1–10 × 10^4 cells within the live gate.

Preparation of CD3/TCR$^-$ Thymocytes. Total thymocytes were coated with hamster anti-CD3 mAb (145-2C11 supernatant) at 4°C for 30 min and then washed 2× in Hanks-buffered saline plus 10% FCS. CD3-coated cells were then allowed to adhere to plastic plates coated with anti-hamster Ig (Fab_2) at room temperature in the presence of sodium azide to prevent receptor internalization. Nonadherent cells were found to be >95% CD3 $(145\text{-}2C11)^-$ and TCR $(H57\text{-}597)^-$ by FCM.

Protein Analysis. For immunoblots, whole-cell lysates of enumerated thymocytes were prepared as described (32). After electrophoresis, proteins were transferred to nitrocellulose and blotted with the appropriate ζ antisera at a 1:200 dilution. Immunoreactive pro-

tein was detected by blotting with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase, by chemiluminescence using the Western Light system (Tropix Inc., Bedford, MA). For metabolic labeling, 100 × 10^6 thymocytes were labeled for 30 min with [^{35}S]methionine (Tran-^{35}S-label, final concentration 1 mCi/ml; ICN Biomedicals Inc., Costa Mesa, CA) in methionine-free RPMI plus 10% fetal calf serum. Labeled cells were washed in PBS and lysed at 4°C in buffer containing 1% NP-40, 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), 20 μg/ml leupeptin, and 40 μg/ml aprotinin. Lysates were then incubated at 4°C overnight in the presence of anti-ζ antiserum 528 and protein A–Sepharose. Immunoprecipitated samples were washed 3× in lysis buffer containing 0.2% NP-40, solubilized by boiling for 5 min in nonreducing SDS loading buffer and analyzed by two-dimensional SDS-PAGE (13%) and autoradiography.

RNA Extraction and Analysis. Thymocyte RNA was extracted as described (33). cDNA probes for Cα (34), Cβ (35), Cγ (36), and Cδ (37) were provided by E. Shevach (NIH), probes for CD3γ (38), CD3δ (39) and CD3ε (40) were provided by M. Wilkenson (Oregon Health Sciencies University, Portland, OR); RAG-1 (41) and RAG-2 (42) probes were provided by D. Schatz (Yale University School of Medicine, New Haven, CT); and ζ cDNA and genomic clones (1, 25) were provided by R. Klausner (NIH). Human β-actin and GAPDH cDNAs were purchased from Clontech Laboratories Inc. (Palo Alto, CA). Probes were labeled with ^{32}P using the random primer method (43).

Results

Transgene Construction. The transgenes generated in these experiments were designed to express either the FL ζ chain or ζ chain variants with only 2 or 1 TAM functional elements (Fig. 1). To produce FL ζ chain, a minigene was created by fusing the 5′ portion of the murine ζ cDNA (encoding exons I-V) with genomic sequences encoding exons VI-VIII (1, 25; see Fig. 1). The assembled minigene therefore contained introns V-VII and the native polyadenylation signal (25). The ζ–CT108 minigene was made by replacing the cDNA fragment with an otherwise identical sequence that contained an inframe termination codon (TAA) in place of Glu^{108} (Fig. 1 *A*). Consequently, this construct should direct the synthesis of a truncated ζ chain that lacks residues 109–164 of the cytoplasmic domain and contains only the first TAM (12) (Fig. 1 *B*). The η minigene was made by replacing the ζ cDNA fragment with the complete coding sequences from murine η cDNA (2; Fig. 1 *A*). As illustrated in Fig. 1 *B*, η chain differs from ζ only in its distal COOH-terminal domain such that the third TAM, as well as a putative nucleotide binding site (1), is destroyed. The assembled minigenes were inserted into the human CD2 promoter/enhancer cassette which has been shown previously to direct high level, T lineage–specific expression of transgenes in mice (26). Transgenic mice were generated by microinjection of constructs into embryos obtained from inbred FVB/N females. A total of seven ζ-FL, six ζ-CT108, and six η transgenic founder lines were obtained and subsequently analyzed.

Expression of the ζ-FL, ζ–CT108, and η Transgenes. Transgene expression was quantitated by hybridizing Northern blots of total thymocyte RNA from control and transgenic mice with a probe derived from 5′ ζ/η cDNA sequences spanning exons I-V (Fig. 2). When compared with the level of endog-

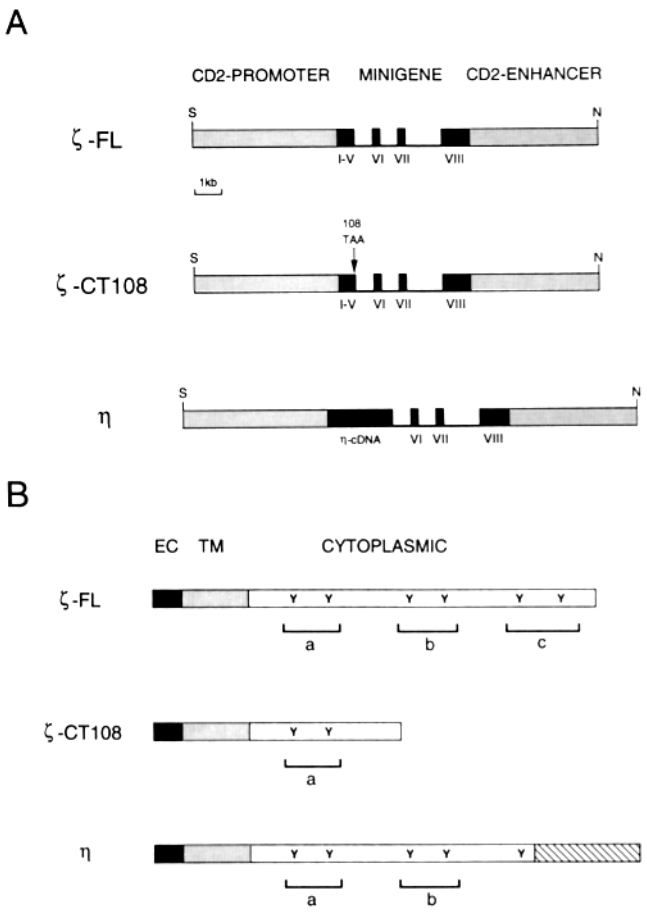

Figure 1. (*A*) ζ-FL, ζ-CT108, and η transgene constructs. Promoterless ζ, CT108, and η minigenes were placed under the control of the human CD2 promoter and 3′ regulatory sequences (*grey boxes*) (26). *Black boxes* represent ζ cDNA, η cDNA, or ζ exons; *lines* represent introns. The location of the Glu → Stop mutation within construct ζ-CT108 is indicated by the *arrow*. S, Sal1; N, Not1 sites. (*B*) Schematic representation of the ζ-FL, ζ-CT108, and η chains. The extracellular (EC), transmembrane (TM), and cytoplasmic domains are depicted as *black*, *shaded*, or *open boxes*, respectively. The *striped box* at the 3′-terminus of η-chain indicates the unique region generated by alternative splicing. The location of TAMs, *a–c* within the cytoplasmic domains of transgenic proteins is indicated. Y, tyrosine residues. Note that the third TAM is destroyed by alternative splicing in η chain.

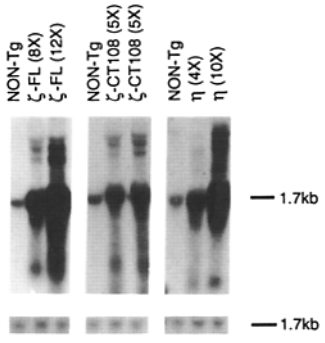

Figure 2. Quantitation of ζ-FL, η, and ζ-CT108 transgene expression. 10 μg of total RNA from thymus was electrophoresed through a formaldehyde/agarose gel, transferred to nylon membranes, and hybridized with a probe derived from the ζ coding region (*top*) or with a probe specific to the 5′-untranslated region of endogenous ζ mRNA (*bottom*). The levels of transgene expression relative to endogenous ζ (determined by densitometry) are shown in parentheses. Non-Tg, nontransgenic littermate.

enous ζ transcripts (1X), transgene expression ranged from 2 to 12× in ζ-FL lines, 1.5 to 10× in ζ–CT108 lines, and 1 to 10× in η lines. In addition, probing of Northern blots with an oligonucleotide specific for the 5′ untranslated region of endogenous ζ mRNA demonstrated that the level of endogenous ζ transcripts was unaffected (Fig. 2).

In thymocytes from ζ-FL transgenic lines, the amount of ζ protein was increased and was commensurate with levels of transgene-encoded ζ mRNA (Fig. 3 *A*). In addition, ζ chains assembled as disulphide linked ζ-ζ homodimers of 32 kD as they did in nontransgenic mice; however, the highest expressing ζ-FL transgenic lines also contained detectable quantities of unassembled ζ monomers of 16 kD (Fig. 3 *A*). In thymocytes from ζ–CT108 and η transgenic mice, transgene expression was determined by immunoprecipitating metabolically labeled material with antisera 528 that was directed against a transmembrane-proximal peptide common to the ζ, CT108, and η proteins (31). Resolution of immunoprecipitates on two-dimensional nonreducing/reducing SDS-PAGE revealed proteins of the predicted size for CT108 (10 kD) (12) and η (23 kD) (2, 31), as well as the presence of all possible dimeric forms between CT108 and endogenous ζ, and between η and endogenous ζ (Fig. 3 *B*). Immunoprecipitation of intact TCRs with anti-CD3ε (mAB 145-2C11) revealed that each of these dimeric forms also assembled with the other TCR subunits into complete TCR complexes (data not shown).

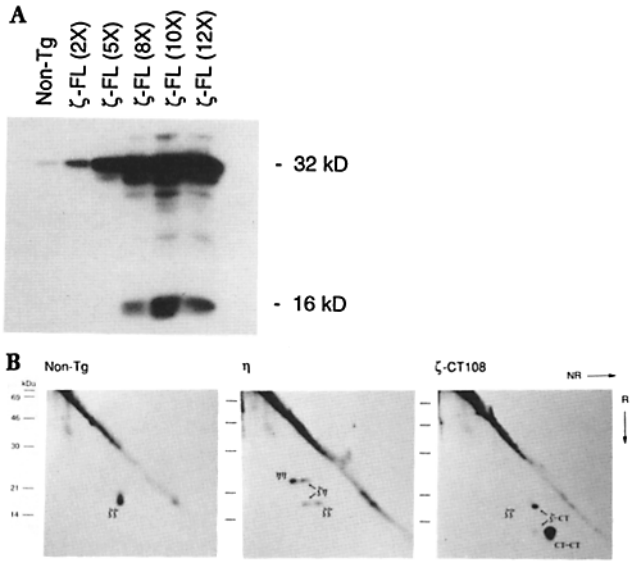

Figure 3. Overexpression of ζ, CT108, and η chains in transgenic thymocytes. (*A*) Immunoblot of thymocyte whole cell lysates (5 × 10^6/lane) from ζ-FL transgenic mice and a Non-Tg littermate resolved by 12% SDS-PAGE under nonreducing conditions. Blotting was performed with anti-ζ antibody 551. The positions of the 32-kD ζ-ζ homodimer and 16-kD ζ chain monomer are indicated. Values in parentheses are estimates of transgene expression relative to endogenous ζ chain and are based on densitometric scanning. (*B*) Two-dimensional nonreducing/reducing SDS-PAGE (13%) of anti-ζ (antibody 528) immunoprecipitates from Non-Tg, η transgenic (line C5160; 5×), and ζ–CT108 transgenic (line C983; 10×) thymocytes. Migration positions of ζ–ζ, ζ–CT108 (CT), CT108–CT108 (CT–CT), ζ–η, and η–η dimers are indicated.

Overexpression of FL ζ chain Alters Normal Thymocyte Development. The effects of transgene expression on thymocyte development were assessed by immunofluorescence and FCM (Fig. 4). In contrast to overexpression of CT108 or η, neither of which inhibited thymocyte maturation at any expression level, overexpression of FL ζ chain had a strikingly inhibitory effect on thymocyte maturation (Fig. 4) as evidenced by: (*a*) decreased thymus size and cellularity; (*b*) reduced numbers of both immature $CD4^+CD8^+$ (double positive [DP]) thymocytes and mature, $CD4^+CD8^-$ and $CD4^-CD8^+$ (single positive [SP]) thymocytes, and most notably, (*c*) reduced numbers of TCR^+ α/β thymocytes (Fig. 4). The degree of maturational arrest correlated with transgene dosage as thymic cellularity and numbers of TCR^+ α/β thymocytes decreased with increasing ζ chain expression (Fig. 4). The highest expressing (10–12×) ζ-FL transgenic lines were essentially devoid of mature SP thymocytes and TCR^+

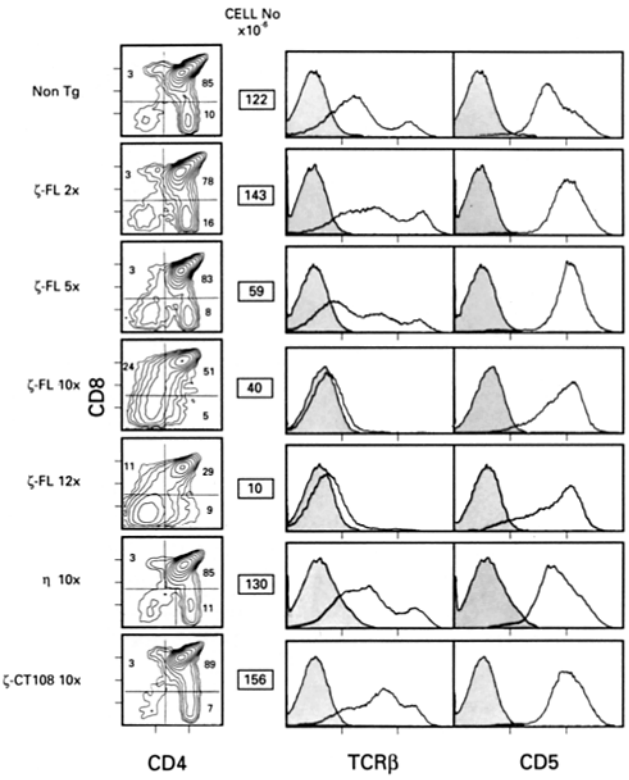

Figure 4. Overexpression of FL ζ but not CT108 or η chain results in thymocyte maturational arrest. Thymocytes from Non-Tg controls, transgenic lines expressing varying levels of the FL-ζ-chain, or from representative ζ–CT108 and η transgenic lines were stained and analyzed by two-color FCM (column *1*) or one-color FCM (columns *2* and *3*). Transgene expression relative to endogenous ζ chain (as assessed by Northern analysis) is indicated within parentheses. Two-color histograms show staining of thymocytes with FITC anti-CD4 vs. biotin anti-CD8 plus TRA. The frequency of $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^+CD8^-$ thymocytes are indicated in their respective quadrants. Total numbers of thymocytes are also shown. Single color histograms were obtained by staining with FITC anti-TCR-β (column *2*) or FITC anti-CD5 (column *3*). Shaded areas indicate staining with control antibody (FITC-Leu 4).

α/β cells (Fig. 4). The cells that were detected within the SP gates in these transgenic lines were $CD4^{lo}$ or $CD8^{lo}$ and represent immature transitional cells that are the immediate precursors of DP thymocytes (44). The unique inhibitory effects of ζ overexpression cannot be attributed to the integration site of the ζ-FL transgene as similar maturational defects were observed in multiple, independently derived transgenic lines (Fig. 4). In addition, since all of the transgenes use the same dominant control element (Fig. 1), nonspecific effects such as variable onset of expression or competition for transcription factors could not account for the differential effects of ζ-FL, ζ–CT108, and η transgenes. Thus, overexpression of the FL ζ chain, but not structurally related molecules such as CT108 or η, specifically inhibited both the generation of TCR^+ α/β thymocytes and the maturation of thymocytes along the CD4/CD8 pathway.

Whereas overexpression of CT108 and the η-chain were not inhibitory, they did affect TCR α/β expression in that thymocytes from ζ-CT108 and η transgenic lines had increased amounts of TCR α/β complexes (Fig. 4, column *2*). Similar results have been reported previously for transgenic mice in which η was expressed under control of the Thy-1 promoter and regulatory elements (45). In fact, thymocytes from lower expressing (2–5×) ζ-FL transgenic lines also exhibited increased TCR α/β expression (Fig. 4). Therefore, CT108, η, and ζ all facilitate increased surface expression of TCR complexes on developing thymocytes, but only ζ overexpression results in developmental arrest and paradoxical loss of TCR expression with increasing transgene dosage.

Transgene Dose-related Suppression of TCR-α and TCR-β Gene Rearrangements in ζ-FL Transgenic Thymocytes. The inhibitory effects of FL ζ chain overexpression on surface TCR α/β expression were entirely unexpected. To understand the molecular basis for decreased TCR α/β expression we determined the abundance of TCR subunit transcripts in total thymocyte RNA from ζ-FL transgenic mice. Whereas the levels of CD3-γ, -δ, and -ϵ transcripts were the same in thymocytes from ζ-FL transgenic and nontransgenic mice (Fig. 5, *right*), the amount of mature TCR-α (1.7 kb) and mature TCR-β (1.3 kb) transcripts were markedly decreased and were inversely related to transgene dosage (Fig. 5, *left*). Because adult thymi from the highest copy number ζ-FL transgenic mice were essentially devoid of TCR^+ α/β thymocytes, we compared the abundance of TCR-α and TCR-β transcripts in transgenic thymocytes to isolated populations of TCR^- thymocytes from nontransgenic mice. In the highest copy number transgenic lines, the amount of mature, 1.7 kb (V_α-J_α-C_α) and mature, 1.3 kb (V_β-D_β-J_β-C_β) transcripts were greatly reduced in comparison to TCR^- nontransgenic thymocytes (Fig. 5). In addition, the TCR-β transcripts that were present in the 8–12× transgenic lines were composed predominantly of immature, 1.0-kb (D_β-J_β-C_β) mRNAs. Conversely, relative amounts of TCR-γ and TCR-δ transcripts increased with increasing transgene expression, approaching the levels observed in TCR^- nontransgenic thymocytes. Thus, overexpression of ζ protein specifically inhibited appearance of mature TCR-α and mature TCR-β transcripts.

Because decreased amounts of mature TCR-α and TCR-β transcripts might have resulted from decreased gene rearrangements, we blotted HindIII- or EcoRI-digested total thymocyte DNA from nontransgenic and ζ-FL transgenic mice with

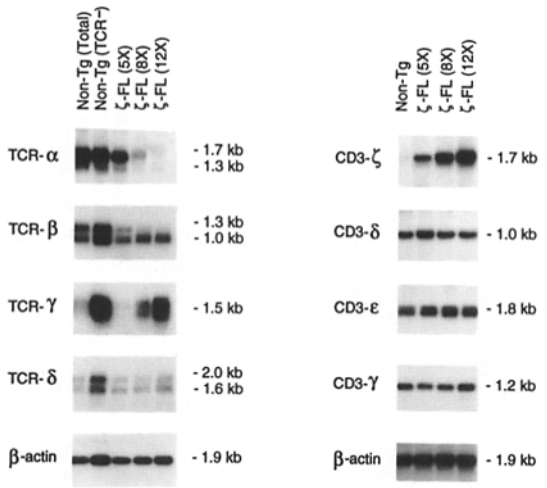

Figure 5. TCR-α and -β transcripts are reduced in ζ-FL transgenic thymocytes. 5 μg of thymocyte RNA from the indicated sources was resolved by formaldehyde/agarose electrophoresis, transferred to nylon membranes and hybridized with radiolabeled probes derived from the various TCR or CD3 cDNAs. Radiolabeled human β-actin cDNA (Clontech Laboratories, Inc.) was used as a control to assess equal loading. (*Left*) Comparison of TCR gene expression in total (lane *1*) or TCR^- (lane *2*) nontransgenic thymocytes with ζ-FL transgenic thymocytes (lanes *3–5*). Relative transgene expression is indicated in parentheses. For TCR-α, -β, and -δ, the sizes of both the mature (larger) and immature (smaller) mRNA species are indicated. (*Right*) Comparison of CD3-$\gamma/\delta/\epsilon$ and ζ transcripts in control (Non-Tg; lane *1*) and ζ-FL transgenic (lanes *2–4*) thymocytes.

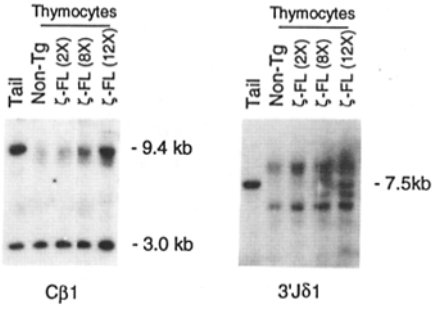

Figure 6. Incomplete rearrangement of the TCR-α and TCR-β genes in ζ-FL thymocytes. 10 μg of total genomic DNA isolated from the indicated sources was digested to completion with HindIII or EcoR1 and analyzed by Southern blotting with probes that detect rearrangements of the TCR-β (Cβ1) (47) or the TCR-α or TCR-δ (3'Jδ1) (46) loci. The Cβ1 probe detects two germline HindIII fragments: a 9.4-kb Cβ1-containing germline band that changes in length after V-D-J rearrangement and a 3.0-kb Cβ2-containing band that is unaltered by V-D-J rearrangement and serves as a control for DNA loading and blotting efficiency. The germline configuration of the TCR-α and TCR-δ loci is represented by a 7.5-kb EcoR1 fragment. The unique bands present in thymocytes from ζ-FL (8 and 12×) mice most likely represent D_δ-D_δ or D_δ-J_δ rearrangements.

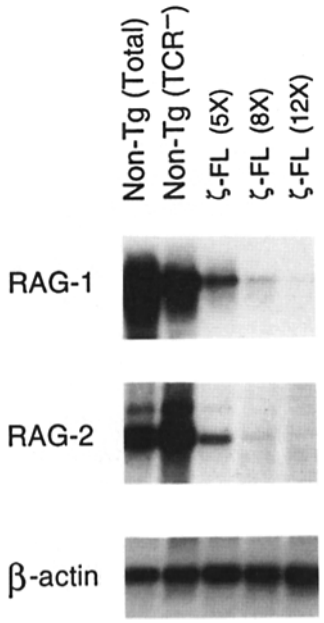

Figure 7. Decreased expression of RAG-1 and RAG-2 in ζ-FL transgenic thymocytes. Northern analysis was performed with 1 μg total thymocyte RNA as described in Fig. 5, legend. Probes consisted of the FL RAG-1 (41) and RAG-2 (42) cDNAs or human β-actin cDNA. Exposure times were 24 h (RAG-1 and β-actin) or 72 h (RAG-2).

probes that specifically detect rearrangements of the TCR α/δ (46) and TCR-β (47) loci. Germline $C_\beta 1$ (9.4 kb) and $J_\delta 1$ (7.5 kb) bands were present in thymocytes from high expressing ζ-FL transgenic mice but not thymocytes from control littermates (Fig. 6), indicating that a high proportion of TCR-β and TCR-α loci have not fully rearranged.

Reduced Levels of RAG-1 and RAG-2 Transcripts in ζ-FL Transgenic Thymocytes. To understand the failure of the TCR-α and TCR-β genes to fully rearrange in thymocytes from high expressing ζ-FL transgenic mice, we examined transgenic thymocytes for the presence of mRNAs encoding products of the recombination activating genes RAG-1 and RAG-2 (41, 42), upon which TCR gene rearrangements are critically dependent (48, 49). Interestingly, RAG-1 and RAG-2 transcripts were reduced in adult thymocyte populations from 5–12× ζ-FL transgenic mice relative either to total thymocytes or to isolated immature TCR⁻ thymocyte populations from nontransgenic control mice (Fig. 7). In addition, RAG-1 and RAG-2 levels were inversely related to transgene dosage (Fig. 7). Thus overexpression of FL ζ resulted in decreased expression of RAG-1 and RAG-2 in developing thymocytes.

Evidence for Activation of an Early Signaling Pathway in ζ-FL Transgenic Mice. TCR-mediated signaling in immature thymocytes is known to result in the rapid disappearance of RAG-1 and RAG-2 transcripts (24). Since ζ can transduce intracellular signals that mimic TCR engagement (13, 16–18), we thought that the unique inhibitory effects of ζ chain overexpression might be a consequence of signaling in early thymocytes. To assess this possibility, we analyzed thymocytes for expression of surface markers that are specifically upregulated in response to TCR-directed signaling. For example, CD5 surface expression is increased in response to either TCR cross-linking or TCR-mediated intrathymic selection events (50, 51). Accordingly, during normal thymocyte development, surface CD5 expression parallels that of surface TCR expression (52) such that $CD3^{lo}$ thymocytes are $CD5^{lo}$, and $CD3^{hi}$ thymocytes are $CD5^{hi}$ (Fig. 8, *left*). In contrast to the concordant expression of TCR and CD5 on normal thymocytes, thymocytes from ζ-FL transgenic mice were predominantly $CD5^{hi}$ despite absence of TCR expression (Figs. 4 and 8). Moreover, high expression of CD5 on TCR⁻ thymocytes was unique to thymocytes from ζ-FL transgenic mice as it was not observed on TCR⁻ thymocytes from normal, SCID, ζ–CT108 transgenic, or η transgenic mice (Fig. 8). ζ-FL transgenic $CD4^-CD8^-CD3^-$ thymocytes also expressed inappropriately high levels of another known signaling molecule, CD2 (Fig. 8) which normally is not expressed at high levels on the cell surface until after complete $V_\beta(D_\beta)J_\beta$ rearrangement (53). Taken together, these data indicate that thymocytes from ζ-FL transgenic mice have undergone intrathymic signaling events despite the absence of TCR α/β expression.

Overexpression of FL ζ Chain Interferes with a Defined Stage of Fetal Thymocyte Development. To determine at what point in thymic ontogeny the inhibitory effects of ζ chain overexpression were first evident, we examined thymocytes from normal and ζ-FL transgenic fetal mice. Maturational defects in fetal thymocytes from ζ-FL transgenic mice, as assessed by decreased thymic cellularity and decreased numbers of DP thymocytes, were evident as early as fetal day 16, but were most pronounced on days 17 and 18 of fetal development (Fig.

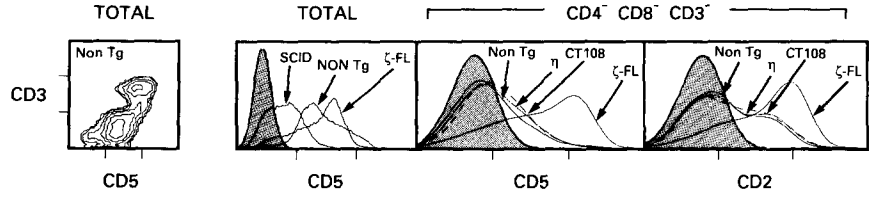

Figure 8. Elevated CD5 and CD2 expression on ζ-FL thymocytes. In the two-color contour plot (panel *1*), thymocytes from adult, Non-Tg FVB/N mice were stained with FITC anti-CD5 vs. biotin anti-CD3 plus TRA to demonstrate the normal relationship between CD5 and TCR surface expression. In the one-color histograms, total thymocytes from Non-Tg, ζ-FL (10×) transgenic, ζ-CT108 (10×) transgenic, η (10×) transgenic, and SCID mice were stained with FITC-anti-CD5 or FITC-anti-CD2. Shaded area represents staining with control antibody, FITC anti-Leu 4. Panel *2* shows CD5 expression on total thymocytes from SCID, Non-Tg, and ζ-FL thymocytes. Panels *3* and *4* show CD5 and CD2 expression, respectively, on $CD4^-CD8^-TCR^-$ thymocytes from Non-Tg, ζ-FL (10×), CT108 (10×), and η (10×) mice. To specifically examine $CD4^-CD8^-TCR^-$ thymocytes, total thymocytes were sequentially stained with FITC anti-CD5 (Panel *3*) or FITC anti-CD2 (panel *4*) followed by a mixture of biotin anti-CD4, biotin anti-CD8, and biotin anti-CD3 plus TRA. CD5 and CD2 expression on $CD4^-CD8^-TCR^-$ thymocytes was then examined by computer-gating on biotin-TRA-negative cells.

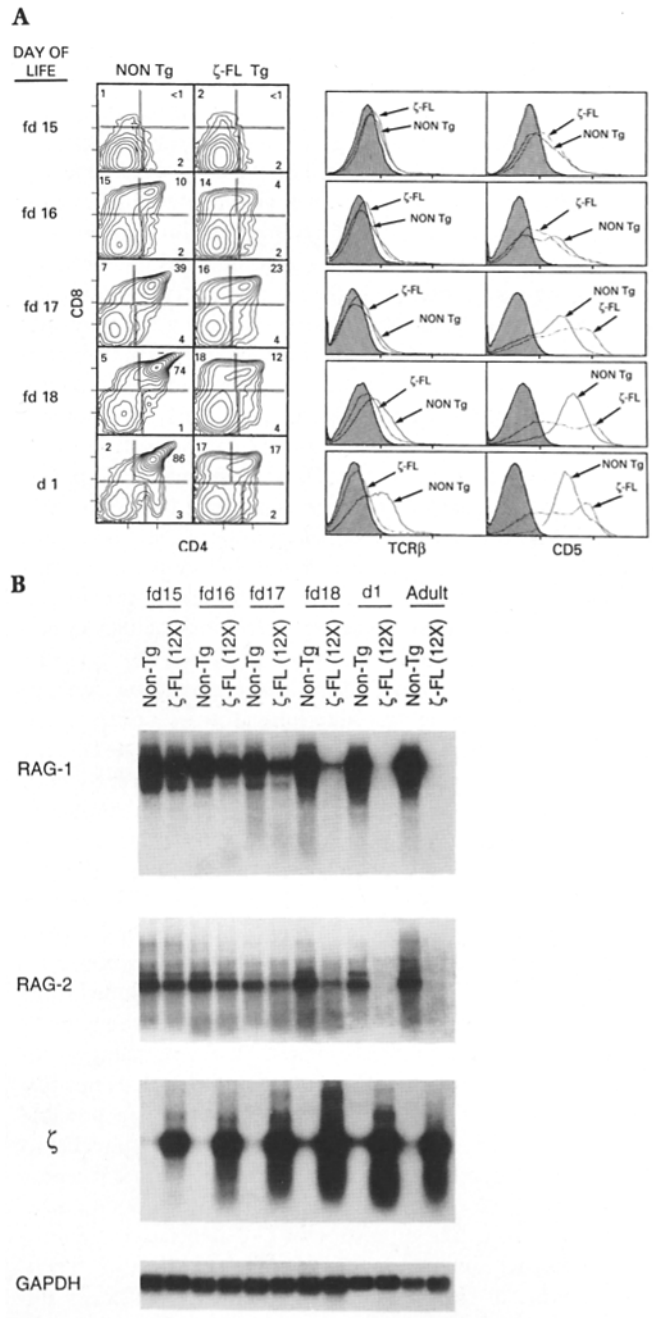

Figure 9. (*A*) Fetal thymocyte development in ζ-FL transgenic mice. Thymocytes were obtained from fetuses generated by timed matings of Non-Tg males and females or ζ-FL (12×) homozygous males and Non-Tg females (ζ-FL Tg). For each timepoint, cells from 25–30 thymi were combined and analyzed by two-color FCM (columns *1* and *2*) or one-color FCM (columns *3* and *4*). Two-color histograms show staining of thymocytes with FITC anti-CD4 vs. biotin anti-CD8 plus TRA. The frequency of $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$ thymocytes are indicated in their respective quadrants. One-color histograms were obtained by staining with FITC anti-TCR-β (column *3*) or FITC anti-CD5 (column *4*). Shaded areas indicate staining with control antibody (FITC-Leu 4). Thymocyte numbers (No. per thymus × 10^{-4}) are averages of multiple independent timed matings: fd15 (Non-Tg: 13.6, n = 105; ζ-FL: 14.3, n = 101); fd16 (Non-Tg: 44.5, n = 109; ζ-FL: 22.1, n = 107); fd17 (Non-Tg: 164.5, n = 98; ζ-FL: 84.5, n = 85); fd18 (Non-Tg: 509, n = 30; ζ-FL: 72.9, n = 24); d1 (Non-Tg: 1136, n = 29; ζ-FL: 122.8, n = 20). (*B*) RAG-1, RAG-2, and ζ expression in fetal thymocytes. Northern analysis was performed with 1 μg of total thymocyte RNA as described in Fig. 5, legend. Probes consisted of murine RAG-1 (41), RAG-2 (42), or ζ-chain cDNAs or human GAPDH cDNA (Clontech Laboratories Inc.). Exposure times were 6 h (RAG-1, ζ, and GAPDH) or 12 h (RAG-2).

9 *A*). Interestingly, although transgene expression begins on or before day 15 of fetal life, RAG-1 and RAG-2 transcripts were initially high and comparable to those in nontransgenic thymocytes on fetal days 15 and 16 but decreased sharply on fetal days 17 and 18 and were essentially absent at birth (Fig. 9 *B*). Atypical $CD5^{hi}TCR^-$ fetal thymocytes were also first detected on fetal day 17 (Fig. 9 *A*). The timing of these events is consistent with accumulation of transcripts that correspond to primarily partial (D_β-J_β) but not complete (V_β-D_β-J_β) TCR-β rearrangements (Fig. 5) as the former are first detectable on fetal days 14 and 15 and the latter on days 16 and 17 (47, 54), and with the near absence of FL TCR-α transcripts (Fig. 5) as these are first detectable on day 17 of gestation (52). These results additionally suggested that ζ-chain overexpression might not affect the development of TCR γ/δ thymocytes because TCR-γ and TCR-δ genes rearrange early in fetal ontogeny and TCR γ/δ^+ cells are detectable by day 15 of fetal development (46). In fact, adult thymi from high expressing ζ-FL transgenic mice that were devoid of TCR^+ α/β thymocytes contained normal numbers of TCR^+ γ/δ thymocytes (not shown), a result consistent with the presence of abundant TCR-γ and TCR-δ transcripts (Fig. 5). Together, these data demonstrate that ζ-mediated downregulation of RAG-1 and RAG-2 occurs at defined point in thymocyte development, namely after rearrangement of the TCR-γ and TCR-δ genes but prior to complete rearrangement of the TCR-α and TCR-β genes.

Discussion

The present study demonstrates that overexpression of FL ζ chain in early TCR^- α/β thymocytes results in downregulation of RAG-1 and RAG-2 transcripts, inhibits complete rearrangement of the TCR-α and TCR-β genes and blocks entry of thymocytes into the CD4/CD8 developmental pathway. These inhibitory effects occur at an identifiable point in fetal ontogeny and are dependent upon overexpression of the intact ζ chain as they are not reproduced by overexpression of either truncated ζ chains (CT108) or a naturally occuring splice variant of ζ (η). Moreover, downregulation of RAG-1 and RAG-2 correlates with increased surface expression of known thymocyte signaling markers such as CD5 and CD2. Thus, these results suggest that an early TCR-independent signaling mechanism that is differentially responsive to individual members of the ζ-family of proteins can regulate expression of RAG-1 and RAG-2. In addition, they provide the first demonstration of different signaling activities by the ζ and η chains.

ζ chain and related members of the ζ-family dimers (η and FcεR1γ) function in various contexts to couple surface structures to intracellular signaling pathways. The recent appreciation that ζ, η, and γ differ with respect to the number of functional signaling motifs (3, 2, or 1, respectively) present

within their cytoplasmic domains has led to speculation that these proteins may play specialized roles in ontogeny, perhaps by coupling surface receptor complexes to different signaling responses. In this study, the transgene encoded ζ, η, and CT108 proteins, which contain identical extracellular and transmembrane domains, each promoted the assembly and transport of intact TCR α/β's to the cell surface. Because these proteins differ only in their cytoplasmic regions, our results suggest that the intact ζ chain cytoplasmic tail but not the cytoplasmic tail of η or CT108, can couple an as yet unidentified surface structure(s) to an early signaling response that regulates expression of RAG-1 and RAG-2. Whether this reflects a specific functional role for 3 TAM repeats or for the third TAM, which also overlaps a consensus sequence for nucleotide binding (1, 12), remains to be determined.

The phenotypic effects of FL-ζ overexpression are essentially identical to those that result from TCR-mediated signaling in immature populations of TCR^+ thymocytes, in that both result in downregulation of RAG-1 and RAG-2 (24) and enhanced surface expression of CD5 (51). The observation that ζ chain can mimic TCR signals is consistent with the previous results of others (13, 16–18), and also demonstrates that in thymocytes, ζ is capable of transducing similar signals regardless of whether they are initiated by cross-linking of the TCR α/β complex or by engagement of other presumably distinct ζ-associated surface structures. In this respect, it is notable that high levels of ζ chain are detectable on the surface of both fetal and adult thymocytes from transgenic mice (our unpublished data).

Whereas the phenotypic effects of ζ overexpression resemble those generated by TCR engagement, they are notably distinct from those that result from overexpression of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, which also participate in T cell signaling (55, 56). Overexpression of $p56^{lck}$ blocks complete TCR-β rearrangement and entry of thymocytes into the CD4/CD8 developmental pathway, but does not inhibit TCR-α rearrangement; and so presumably does not terminate RAG expression (56). On the other hand, early overexpression of $p56^{fyn}$ renders thymocytes more responsive to TCR-mediated stimulation but does not inhibit normal development (55). That premature downregulation of RAG-1 and RAG-2 is observed only in mice that overexpress FL-ζ chain suggests that ζ may act as a critical intermediate to couple surface structures to a signaling response that controls expression of these transcripts.

Our results do not exclude the possibility that ζ chain overexpression results in signaling independent of either surface expression or association with other surface components. For example, it is conceivable that overexpression of ζ could result in intracellular signaling during transit through the endoplasmic reticulum and Golgi apparatus; however, spontaneous signaling in established cell lines has not been reported in association with overexpression of either ζ or chimeric molecules that contain the intact ζ cytoplasmic domain (16, 17). It is also possible that the FL ζ chain mediates its effects by binding signaling intermediates such as protein tyrosine kinases via its cytoplasmic tail thereby inhibiting the transduction of intracellular signals. However, such an effect is inconsistent with the observed increase in expression of signaling markers such as CD2 and CD5. Consequently, we think it more likely that in early thymocytes, ζ couples surface complex(es) other than the intact TCR to an intracellular signaling pathway that can regulate expression of RAG-1 and RAG-2. Various alternative signaling molecules such as Thy1 and CD2 are expressed on fetal thymocytes and are thought to function in early stages of thymocyte development. In mature T cells, these molecules can transduce intracellular signals when expressed in conjunction with TCR (57, 58). Interestingly, although truncated ζ chain variants are capable of coupling the TCR to signal transduction pathways, coexpression of TCRs that contain FL ζ chain is required to facilitate efficient signaling by Thy1 (12–14) and CD2 (14). It has recently been proposed that CD16 (FcγRIII) may serve a role similar to the TCR in fetal thymocytes by coupling accessory molecules such as Thy1 and CD2 to signal transduction pathways (59). CD16, which is expressed prior to the TCR α/β in fetal ontogeny, associates with dimers composed of ζ and/or FcϵR1γ chains, but assembly with γ chains is strongly favored (10). Consequently, ζ may be effectively excluded from associating with CD16 by γ during normal thymocyte development, resulting in surface expression of a signaling structure that does not contain ζ–ζ dimers and that does not terminate RAG-1 and RAG-2 expression. Indeed, a possible function for the early expression of γ and η may be to specifically compete with ζ for assembly into surface receptor complexes. Overexpression of ζ in transgenic mice could instead result in surface expression of ζ–ζ-associated signaling complexes that can downregulate RAG-1 and RAG-2. Thus, the outcome of thymocyte development may critically depend upon competition between members of the ζ-family dimers for assembly with surface receptor complexes that are expressed on early thymocytes.

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