Delayed Maturation of CD4$^-$CD8$^-$ FcγRII/III$^+$ T and Natural Killer Cell Precursors in FcεRIγ Transgenic Mice

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Summary

FcεRIγ (γ) is a member of a group of related proteins (the ζ-family dimers) that function as signal-transducing components of both Fc receptors and the T cell antigen receptor (TCR). Analysis of γ expression during fetal thymus ontogeny revealed that it is expressed in early thymocytes, before the initiation of clonotypic TCR-α and TCR-β gene rearrangement but is down-regulated in most adult thymocytes. To explore a possible role for γ in thymocyte development, we generated transgenic mice in which this protein was overexpressed at all stages of ontogeny. Overexpression of γ inhibited the maturation of T cells as well as natural killer (NK) cells. The developmental effects were transgene dose related and correlated with markedly delayed maturation of fetal CD4$^-$CD8$^-$ FcRII/III$^+$ thymocytes, cells thought to include the progenitors of both T and NK cells. These results suggest that the ζ and γ chains serve distinctive functions in thymocyte development and indicate that Fc receptor(s) may play an important role in regulating the differentiation of early progenitor cells within the thymus.

Signals transduced by the TCR regulate the stage-specific development and selection (positive and negative) of thymocytes (1, 2). The signal-transducing potential of the TCR complex is conferred by multiple subunits (the CD3-γ, -δ, and -ε, and the ζ, η, or FcεRIγ chains) that share a conserved structural element, the immunoreceptor tyrosine-based activation motif (ITAM)[1] (3). A considerable body of evidence supports the current notion that ITAMs, through their ability to facilitate activation of cytoplasmic protein tyrosine kinases, mediate all known TCR effector functions (4).

The signals that regulate the earliest stages of thymocyte maturation are less understood. Precursor CD4$^-$CD8$^-$TCR$^-$ thymocytes populate the murine thymus on or about day 12 of gestation (2). These early thymocytes, which are thought to include the precursors of both T cells and NK cells (5, 6) progress through multiple stages of differentiation before expressing CD4, CD8, and α/βTCR (2, 7). Although the molecular events that regulate their transit through these various stages remain uncharacterized, recent evidence suggests that they may receive activating signals through cell surface structures (8). The majority of early fetal thymocytes express low affinity receptors for IgG (FcγRII and/or FcγRIII) (5). FcγRIII is also expressed on mature NK cells where it functions as a signal-transducing complex to mediate antibody-dependent cellular cytotoxicity (ADCC), and on a subset of mature T cells (9). These observations have led to speculation that FcRIII may represent a developmentally important signaling structure in early CD4$^-$CD8$^-$TCR$^-$ thymocytes (5).

In mice, the FcγRIII α chain forms a complex with a dimer composed of FcεRIγ chains (10, 11). Initially identified as a subunit of the high affinity IgE receptor, FcεRIγ is also a subunit of the high affinity IgG receptor (FcγRI), and in a subset of T cells, functions as a subunit of the TCR complex (12–17). The structural and functional similarity between FcεRIγ and ζ suggests that these proteins are members of a family of signal-transducing proteins whose genes were likely generated by duplication (10, 18). Although FcεRIγ is reportedly expressed in early thymocytes, its potential role in thymocyte development remains controversial. The observation that T cell development is unaffected in mice lacking FcεRIγ argues that its

[1] *Abbreviations used in this paper* ADCC, antibody-dependent cellular cytotoxicity; DN, double negative; DP, double positive; FCM, multicolor flow cytometry; fd, fetal day; γTg, γ transgenic; ITAM, immunoreceptor tyrosine-based activation motif; poly(IC), polyinosinic-polycytidylic acid.

function is not critical for T cell maturation (19). However, recent experiments indicate that Fcγ receptors on fetal thymocytes may transduce developmentally important signals (20). In this study, we document that FcεRIγ is expressed early in fetal thymic ontogeny, before the rearrangement and expression of the clonotypic TCR-α and TCR-β chains. To investigate the role of FcεRIγ in thymocyte development, we generated transgenic mice that express elevated levels of FcεRIγ at all stages of fetal ontogeny. We found that overexpression of FcεRIγ inhibited the maturation of both T cells and NK cells. The developmental effects were transgene dose related and were correlated with delayed maturation of fetal FcγRII/III$^+$ thymocytes into FcγRII/III$^-$ thymocytes. Collectively, these results show that FcεRIγ can regulate the differentiation of an early progenitor cell population within the thymus.

Materials and Methods

DNA Constructs and Microinjection. A 4.8-kb BclI genomic fragment containing the human FcεRIγ (γ) promoter and coding sequences (18) was cloned into the BamHI site of plasmid CD2-B-N2 (21) after removal of the SalI-BamHI fragment containing the human CD2 promoter and minigene. A 9.8-kb fragment composed of the γ gene and the human CD2 3-prime enhancer was then generated by digestion with NotI, separated from vector sequences by gel electrophoresis, purified as described (22), and used for pronuclear injection. Transgenic mice were identified by Southern blotting of tail DNA using the human CD2 enhancer sequences as a probe.

Mice. Transgenic mice were generated by injection of zygotes obtained from matings of inbred FVB/N mice. Timed matings were performed with either non Tg FVB/N males × non Tg C57BL/6J females or homozygous γ551Tg FVB/N males × non Tg C57BL/6J females. The first day of mating was designated as day 0.5. FVB/N × C57BL/6J matings were performed because staining with mAbs 2.4G2 (FcγRII/III) and PK136 (NK1.1) were determined to be superior in the C57BL/6J background; however, the developmental effects of the γTg were identical regardless of the background.

RNA Extraction and Analysis. Purification of total thymocyte RNA and Northern blot analysis were performed as described previously (22). cDNA probes for TCR-α (Cα; 23) and TCR-β (Cβ; 24) were provided by E. Shevach (National Institutes of Health [NIH]). ζ cDNA was provided by R. Klausner (NIH). Human (18) and murine (10) FcεRIγ cDNAs were isolated as described. Human GAPDH cDNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Antibodies and Reagents. mAbs used for flow cytometric analysis included: FITC conjugated anti-Thy-1.1 (OX-7), anti-Thy 1.2 (53-2.1), anti-CD4 (RM4.5), anti-TCR-β (H57-597), anti-CD8α (53-6.7), anti-CD3ε (145-2C11), anti-CD2 (RM2.5), anti-CD25 (7D4), and anti-HSA (M1/69); PE-conjugated anti-CD4 (RM4.5), anti-CD8α (53-6.7), anti-CD3ε (145-2C11), anti-B220 (RA3-6B2), and anti-NK1.1 (PK136); and biotinylated anti-FcγRII/III (2.4G2), anti-CD8α (53-6.7), anti-CD4 (RM4.5), anti-B220 (RA3-6B2), anti-CD3ε (145-2C11), anti-CD5 (53-7.3). Unconjugated mAb 2.4G2 was additionally used to block nonspecific binding and for immunoprecipitation. All of the aforementioned antibodies were purchased from PharMingen (San Diego, CA). Streptavidin-PE (PharMingen) and streptavidin-Red 670 conjugate (GIBCO-BRL, Gaithersburg, MD) were used as second step reagents. Biotin or FITC-conjugated anti-human CD3 (mAb SK7; Becton Dickinson and Co., San Jose, CA) were used as control antibodies at a concentration of 10 μl/10^6 cells. Anti-FcεRIγ was generated in rabbit to a peptide corresponding to amino acids 80–86 of human FcεRIγ (25). Rabbit anti-rat sera were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Multicolor Flow Cytometry (FCM). Single cell suspensions (10^6 cells/ml) of thymi or LN were washed twice in PBS containing 0.5% BSA and 0.05% sodium azide before and after each incubation with antibody. Cell suspensions were preincubated with unconjugated mAb 2.4G2 to inhibit nonspecific staining unless cells were to be stained for FcγRII/III. Incubations with labeled mAb (10 μg/ml), streptavidin-PE or streptavidin-Red 670 were performed at 4°C for 30 min. FCM was performed using a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and data were analyzed using FACScan® research software. Unless stated otherwise, fluorescence data were collected on 5 × 10^4 cells, gating on viable cells as assessed by forward and side scatter profiles. In Fig. 6, *B* and *C*, gates were set on specific thymocyte subpopulations at the time of collection and data were collected on 4–5 × 10^4 viable cells. Data were displayed as logarithmic overlay histograms or dot plots.

Protein Analysis. Thymocytes were lysed in buffer (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 20 μg/ml leupeptin, 40 μg/ml aprotinin) containing 1% NP-40 (adult thymocytes) or 1% digitonin (fetal thymocytes), precleared with protein A–Sepharose plus normal rabbit serum, then incubated with protein A–Sepharose plus 10 μl antisera. Immunoprecipitates were washed, solubilized in loading buffer + 2ME, and resolved by 14% SDS-PAGE. Separated proteins were then transferred to nitrocellulose and blotted with a 1:1,000 dilution of anti-γ. Detection of immunoreactive proteins was done by chemiluminescence using the ECL system (Amersham Corp., Arlington Heights, IL).

Cytotoxicity Assays. Mice were injected with 100 μg of polyinosinic-polycytidylic acid (poly[IC]) (Sigma Chemical Co., St. Louis, MO). Cytotoxicity assays were performed with splenocytes obtained 36 h after injection. Target cells were labeled with ^{51}Cr (Amersham Corp.) at 100 μCi/10^6 cells for 1 h at 37°C, washed twice, and used in a standard 6 h ^{51}Cr-release assay with 2,500 target cells/well in 96-well V-bottom plates. Lysis was performed in RPMI 1640 medium containing 10% heat-inactivated FCS, 10 mM Hepes, and 500 U/ml human IL-2 (Hoffman-LaRoche, Nutley, NJ). NK activity was evaluated on the NK-sensitive target cell line, YAC-1. ADCC assays were performed essentially as described (5) with the exception that 3 mM rather than 30 mM 2,4,6-trinitrobenzyl sulfonic acid was used.

Results

Differential Expression of ζ and FcεRIγ during Fetal Ontogeny. To examine the normal pattern of FcεRIγ (γ) expression during ontogeny, Northern blot analysis was performed on RNA obtained from thymi on sequential days of fetal development. γ transcripts were present on fetal day 13.5, the earliest stage in which the thymus could clearly be identified in situ (Fig. 1). γ mRNA levels were highest at early stages of development (fetal days 13.5–16.5), decreased progressively during late gestation, and were barely detectable in adult thymocytes (Fig. 1). In contrast, low levels of ζ mRNA were first detectable in the thymus on

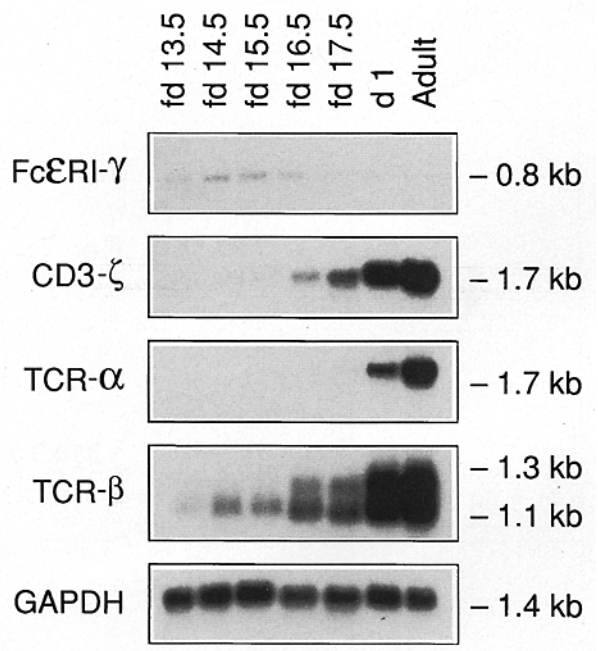

Figure 1. Expression of γ and ζ transcripts in fetal thymus. 5 μg of total thymocyte RNA from sequential days of gestation was resolved by formaldehyde/agarose electrophoresis, transferred to nylon membranes, and hybridized with radiolabeled probes derived from γ, ζ, TCR-α, or TCR-β cDNAs. Radiolabeled human GAPDH cDNA was used as a control to assess equal loading. For TCR-α, the size of the mature (1.7-kb) transcript is indicated. For TCR-β, sizes of both the immature (1.0-kb) and mature (1.3-kb) transcripts are indicated. Exposure times: ζ and TCR-β, 12 h, TCR-α, 24 h; γ, 48 h.

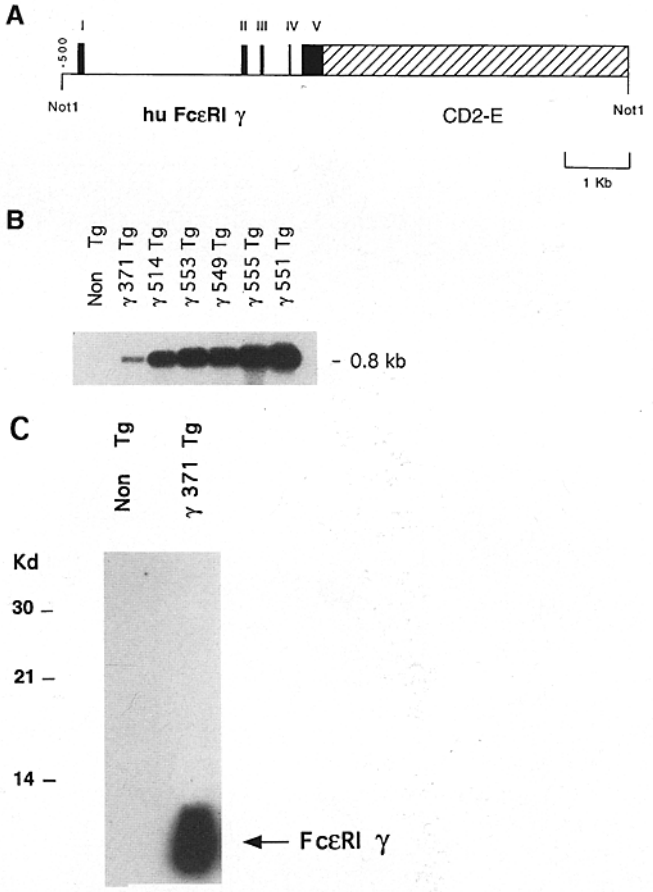

Figure 2. (*A*) Diagrammatic representation of the human (*hu*) FcεRIγ transgene. A genomic DNA fragment containing the human γ promoter and coding sequences was ligated to the human CD2 3-prime enhancer element (*hatched box*, reference 21). Black boxes represent exons (I–V); lines represent introns. The NotI fragment was purified from vector sequences and used for microinjection. (*B*) Quantitation of γ transgene expression. 5 μg of total RNA from adult thymus was electrophoresed through a formaldehyde/agarose gel, transferred to nylon membrane, and hybridized with a probe derived from human γ cDNA. Transgene expression in the six founder lines is compared with that of nontransgenic (*Non Tg*) mice. The transgene copy number for each founder line (as assessed by Southern blotting) was: γ371 (2), γ514 (5); γ553 (8), γ549 (12), γ555 (25), γ551 (35). For RNA quantitation, membranes were stripped and reprobed with murine γ cDNA (not shown). (*C*) Detection of transgenic γ chain in thymocytes by immunoblot. Thymocytes (10^8) from adult Non Tg or γ transgenic (founder line γ514) mice were lysed, precleared, then incubated with protein A–Sepharose plus 10 μl anti-γ. Immunoprecipitates were washed, solubilized in loading buffer and resolved by 14% SDS-PAGE. After transfer, nitrocellulose membranes were incubated with anti-γ followed by goat anti–rabbit horseradish peroxidase and proteins were visualized by chemiluminescence.

days 14.5–15.5 of fetal development and increased markedly during gestation to high levels in adult thymocytes (Fig. 1). The pattern of ζ expression coincided temporally with rearrangement and surface expression of the clonotypic (TCR-α and TCR-β) chains (Fig. 1), an observation which is consistent with the central role of ζ in α/β T cell receptor (α/β TCR) surface expression and signal transduction (26–29). On the other hand, γ predominates in fetal thymocytes during stages that precede expression of the clonotypic TCR-α and TCR-β genes (Fig. 1). The striking difference in expression of γ and ζ during ontogeny suggested that these proteins might play distinctive roles in thymocyte development. To investigate this possibility, we perturbed the normal pattern of γ expression by generating transgenic mice in which γ is overexpressed in early thymocytes and continues to be expressed throughout development.

Generation of FcεRIγ Transgenic Mice. The FcεRIγ (γ) transgene was generated from a 4.8-kb BclI genomic fragment containing the human γ promotor and coding sequences and a 5-kb genomic fragment containing the human CD2 3′-enhancer element (18, 21; Fig. 2 *A*). Six independently derived γ transgenic (γTg) founder lines were obtained by microinjection of DNA into embryos derived from inbred strain FVB/N females.

Transgene expression was detected by Northern blotting of total thymocyte RNA with radiolabeled human γ cDNA. RNA expression correlated with transgene copy number, varying ~12-fold between the lowest (γ371) and the highest (γ551) expressing founder lines (Fig. 2 *B*). Both transgenic and endogenous (murine) transcripts were expressed early in fetal ontogeny; however, unlike endogenous γ mRNA, high level expression of the transgene continued throughout thymocyte development (not shown). The level of

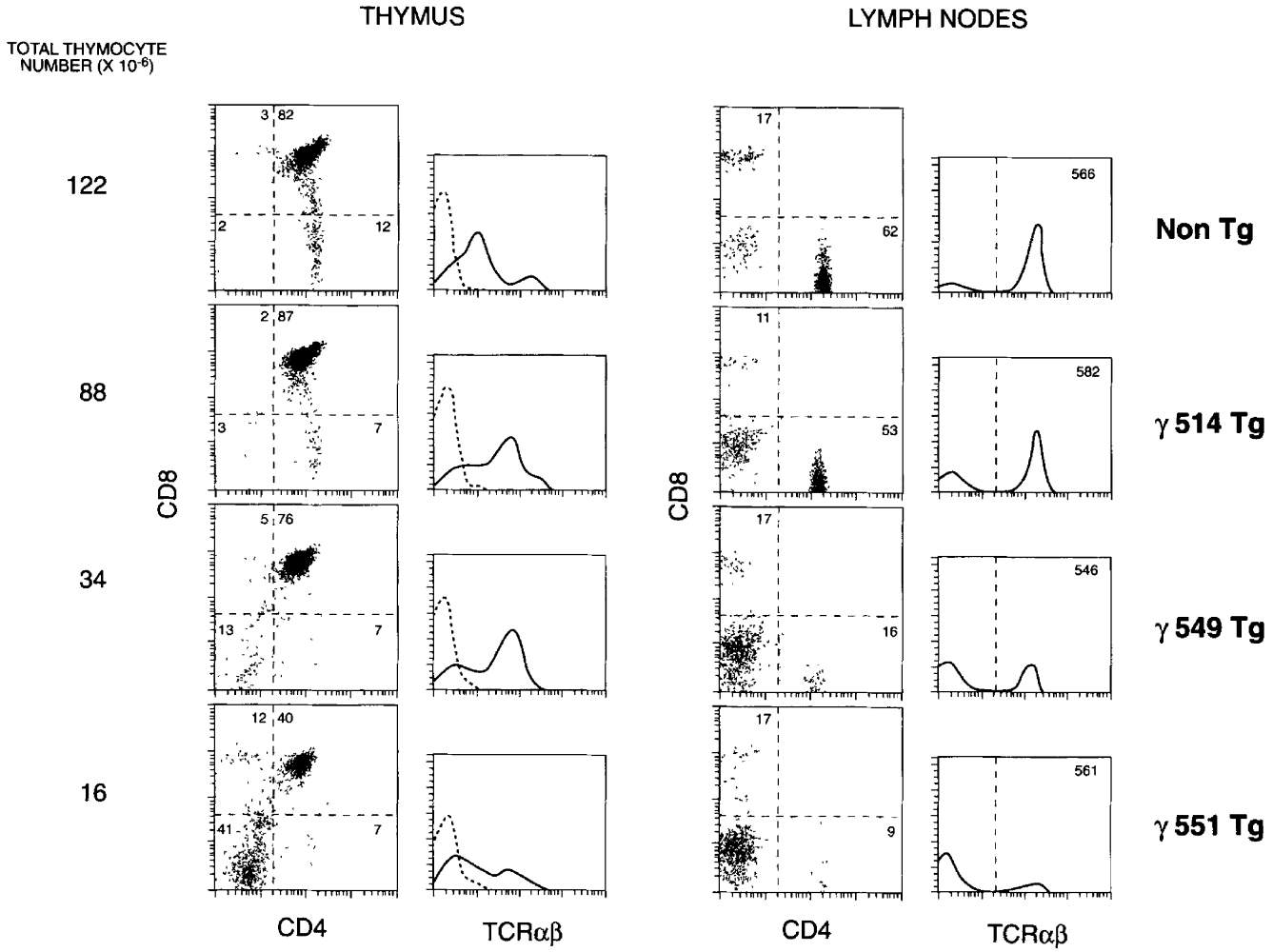

Figure 3. Overexpression of γ chain impairs thymocyte development. Thymocytes (*left*) and LN cells (*right*) from 4-wk-old nontransgenic (Non Tg) mice or from 4-wk-old γ transgenic lines (γ514, γ549, γ551) expressing varying levels of human γ chain were stained and analyzed by two or one color FCM. Two-color plots (columns 1 and 3) show staining of cells with FITC anti-CD4 vs. biotin anti-CD8 plus avidin-PE. The frequencies of $CD4^-CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^+CD8^-$ thymocytes, and $CD4^-CD8^+$ and $CD4^+CD8^-$ LN T cells are shown within their respective quadrants. Numbers on the left show total numbers of thymocytes for each line and are representative of multiple samples. Single-color plots (columns 2 and 4) were obtained by staining with FITC anti-TCR-β mAb. Dotted lines indicate staining with control antibody. For lymph node plots, numbers shown represent the mean fluorescence on TCR-β^+ cells

transgene expression relative to endogenous γ was assessed by sequential hybridization of northern blots with human and mouse cDNA probes followed by densitometric quantitation. Transgene expression was estimated to be 50-fold (Tg line γ371) to 600-fold (Tg line γ551) greater than endogenous in adult thymocytes. In fetal day 15.5 thymocytes, the relative increase was less due to the higher level of endogenous γ; 5-fold in Tg line γ371 and 60-fold in Tg line γ551 (not shown).

Immunoblotting with antiserum that detects both human and murine γ chains demonstrated the presence of human γ chain in adult thymocytes from Tg mice (Fig. 2 *C*). After prolonged exposure, a far less abundant protein of identical mobility, representing endogenous murine γ, was also observed in thymocytes from nontransgenic mice (not shown). Given the high level of sequence conservation between human and murine γ chains (18) it was expected that these proteins would be functionally equivalent. Consistent with this idea, transgene-encoded human γ chains assembled both as homodimers and as heterodimeric forms with endogenous murine ζ chain (not shown). Analysis of proteins from both adult and fetal (day 17.5) thymocytes by two-dimensional SDS-PAGE revealed that both ζγ and γγ dimers were present in thymocytes from γTg mice and their relative abundance predictably increased with transgene copy number. In contrast, ζγ and γγ dimers were virtually undetectable in adult nontransgenic mice (not shown).

Dose-related Effects of the γ Transgene on Thymocyte Development. To determine the effect of γ overexpression on T cell development, we first analyzed the phenotype of adult thymocytes from control and γTg mice. Thymocyte numbers were reduced in γTg mice (Fig. 3; Table 1). Moreover, the effect of γ overexpression on thymus cellularity was transgene dose dependent as manifested by the

Table 1. *Numbers of Thymocytes in FcεRIγ Transgenic (γ Tg) Mice*

	Non Tg	γ514 Tg	γ551
	$n = 6$	$n = 4$	$n = 5$
Total	160 ± 21	109 ± 31	17 ± 5
$CD4^-CD8^-$	2.6	3.6	7.0
$CD4^+CD8^+$	138	96	8.0
$CD4^+CD8^-$	14.2	5.0	1.0
$CD4^-CD8^+$	5.1	3.3	1.5

Data are expressed in 10^6 cells. Total thymocytes are given as means ± SEM.

progressive reduction in thymocyte number with increasing transgene copy number (Fig. 3; Table 1). Analysis of CD4 and CD8 expression on thymocytes from γTg mice revealed an impairment of thymocyte maturation. Low copy number transgenic lines exhibited minimal developmental defects whereas high copy number transgenic lines exhibited a progressive loss of mature $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive, SP) and $CD4^+CD8^+$ (double positive, DP) thymocytes and a relative increase in the number of immature $CD4^-CD8^-$ (double negative, DN) thymocytes (Fig. 3; Table 1). γ overexpression also resulted in a corresponding reduction in the number of peripheral T cells (Fig. 3). Both DP and SP thymocytes from γTg mice expressed α/βTCR (Fig. 3). In fact, though reduced in number, DP thymocytes from adult γTg mice expressed higher levels of α/βTCR than DP thymocytes from non-Tg mice (Fig. 3, data not shown). Moreover, despite their reduced numbers, peripheral $CD4^+$ and $CD8^+$ T cells from γTg mice expressed α/βTCR at levels comparable to controls (Fig. 3).

Overexpression of γ did not result in complete developmental arrest, as all thymocyte subsets (DN, DP, and SP) as well as peripheral T cells were present in adult γTg mice. The phenotype of adult γTg mice additionally suggested a block in early thymocyte development. Numbers of DP thymocytes were markedly reduced in high copy number (γ551) Tg mice (DN/DP ratio of 1.0 ± 0.26; $n = 5$ compared to 0.03 ± 0.01; $n = 5$ for controls). In contrast, the transition of DP thymocytes into mature, SP thymocytes appeared less affected as the SP/DP ratio in γ551 Tg mice (0.16 ± 0.05; $n = 5$), was identical to that of control mice (0.11 ± 0.03; $n = 5$). Interestingly, although both $CD4^+$ $CD8^-$ SP and $CD4^-CD8^+$ SP thymocyte numbers were reduced in γTg mice, $CD4^+CD8^-$ thymocytes appeared to be more severely affected (Fig. 3; Table 1). A skewed ratio of $CD4^+CD8^-$ to $CD4^-CD8^+$ cells was also seen in the lymph nodes of γTg mice indicating that the development of $CD4^+CD8^-$ T cells may be selectively impaired (Fig. 3).

γ Overexpression Delays Maturation of α/βTCR$^+$ T Cells. To determine when the developmental impairment was first evident in γTg mice we next analyzed progressive stages of fetal thymocyte maturation. Examination of fetal thymocytes from high copy number (γ551) Tg mice revealed an early perturbation of thymocyte maturation (Figs. 4 and 5). Total numbers of thymocytes were equivalent in control and γ551 Tg mice on day 15, but at all subsequent stages, the number of thymocytes in γTg mice was reduced relative to nontransgenic mice (Fig. 4). By fetal day 17.5, significant numbers of "transitional," $CD4^-CD8^{lo}$ and DP thymocytes were detectable in normal mice, whereas most thymocytes in the γ551 Tg mice remained DN (Fig. 5 *A*). DP and SP thymocytes began to accumulate in γ551 Tg mice only after birth and the relative number of DN thymocytes remained high even in adult mice (Figs. 3 and 5). Thymocytes from γ551Tg mice were immature relative to nontransgenic mice of the same gestational age by several other criteria. For example, most thymocytes from γ551 transgenic newborn mice resembled fetal thymocytes in that they were IL2-Rα$^-$ and expressed low levels of surface CD2, CD5, and α/βTCR (Fig. 5 *B*).

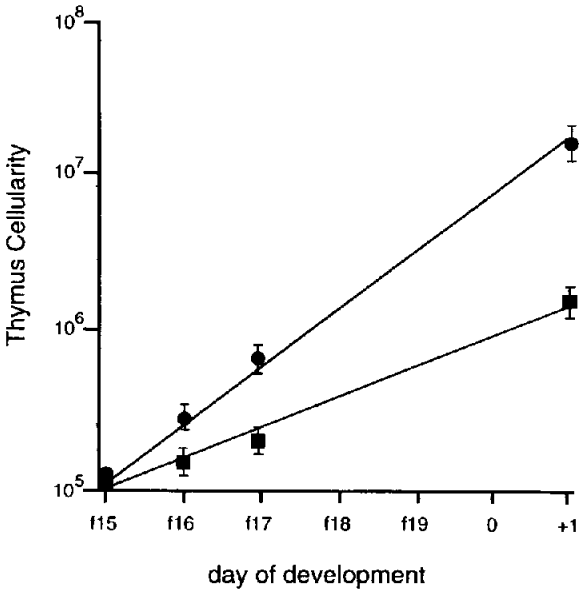

Figure 4. Thymocyte number vs. gestational age Non Tg mice (*circles*) γ551 Tg mice (*squares*). Datapoints represent the mean ± 1 SD obtained from multiple thymi. Numbers of thymocytes in Non Tg and γTg mice were equivalent at fetal day 15.5.

Fetal Thymocyte Development Is Delayed at the $CD4^-CD8^-$ FcγRII/III$^+$ Stage in γTg Mice. Since our analysis of fetal thymocytes from γTg mice revealed a block at the DN→DP transition stage, we examined the phenotype of early DN thymocyte populations in γTg mice. Four distinct subsets of precursor "triple negative" ($CD3^-CD4^-CD8^-$) thymocytes have been identified on the basis of CD44 and CD25 expression that represent progressive stages of development: $CD44^+CD25^-$→$CD44^+CD25^+$→$CD44^{lo/-}CD25^+$→$CD44^-CD25^-$ (30). FACS® analysis of DN thymocyte subsets from newborn γTg mice revealed a specific block in development at the $CD44^{+/lo}CD25^+$ stage (Fig. 5 *C*). The reduction in $CD44^-CD25^-$ triple negative thymocytes was

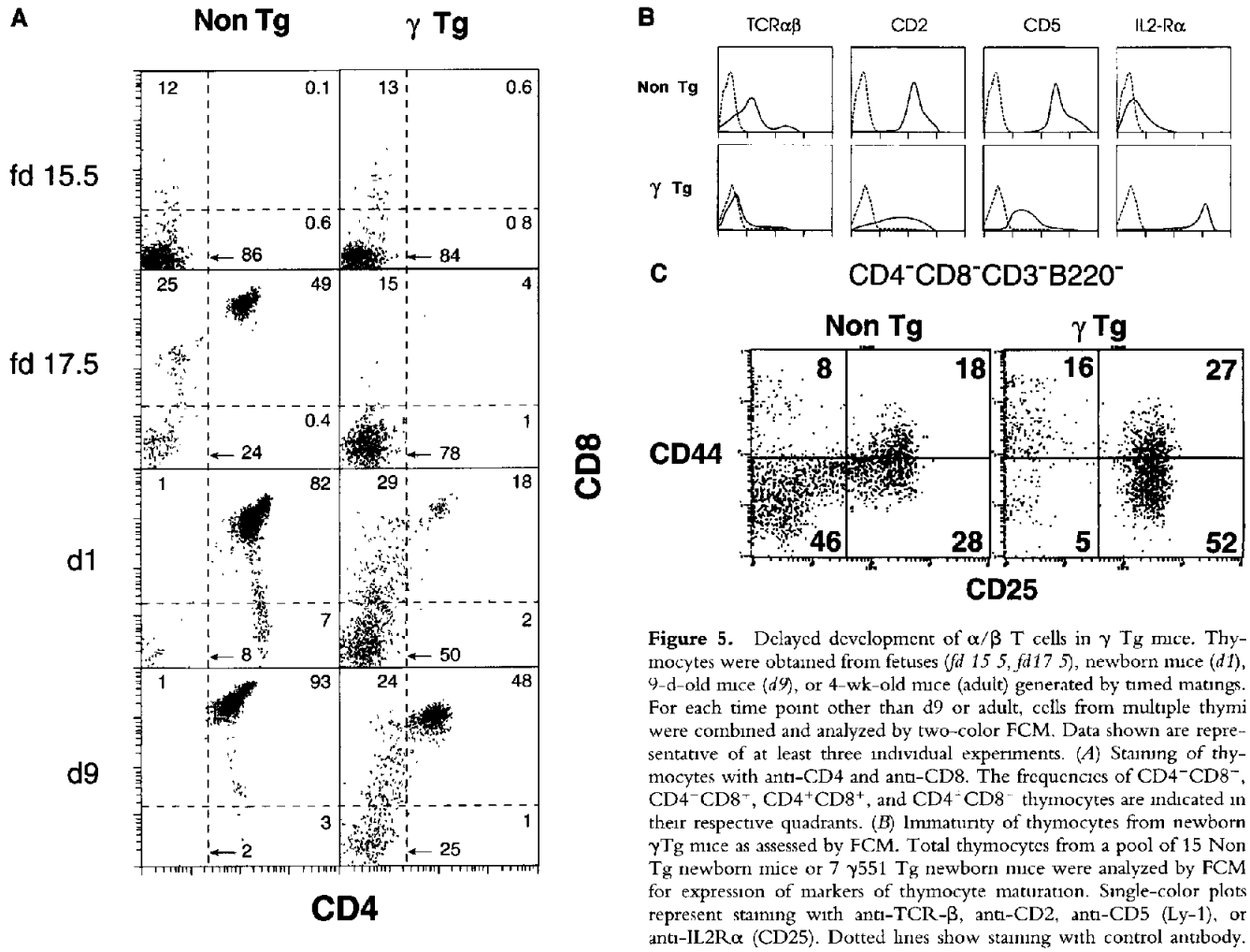

Figure 5. Delayed development of α/β T cells in γ Tg mice. Thymocytes were obtained from fetuses (*fd 15 5, fd17 5*), newborn mice (*d1*), 9-d-old mice (*d9*), or 4-wk-old mice (adult) generated by timed matings. For each time point other than d9 or adult, cells from multiple thymi were combined and analyzed by two-color FCM. Data shown are representative of at least three individual experiments. (*A*) Staining of thymocytes with anti-CD4 and anti-CD8. The frequencies of $CD4^-CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^+CD8^-$ thymocytes are indicated in their respective quadrants. (*B*) Immaturity of thymocytes from newborn γTg mice as assessed by FCM. Total thymocytes from a pool of 15 Non Tg newborn mice or 7 γ551 Tg newborn mice were analyzed by FCM for expression of markers of thymocyte maturation. Single-color plots represent staining with anti-TCR-β, anti-CD2, anti-CD5 (Ly-1), or anti-IL2Rα (CD25). Dotted lines show staining with control antibody. (*C*) Comparison of $CD4^-CD8^-$ thymocyte subsets in newborn Non Tg and γ551 Tg mice. Total thymocytes were stained with biotinylated anti-CD3, -CD4, -CD8, -B220 plus avidin-R670, FITC-anti-CD44, and PE-antiCD25. CD44 and CD25 surface expression was determined on CD3-, 4-,8-, B220- (mix) negative thymocytes by gating. Mix-negative thymocytes were determined to be Thy 1.2^+ by separate staining.

consistent with the low numbers of DP thymocytes observed in γTg mice as these cells are the immediate precursors of DP thymocytes (30). Since γ chain is known to associate with FcγRIII we next examined expression of Fcγ receptors on early thymocyte populations. Staining of fetal day 15.5 thymocytes with mAb 2.4G2 (31), which recognizes the $FcγRIIb_1$, $FcγRIIb_2$, and FcγRIII receptor isoforms, revealed that the majority of thymocytes from control and γ551 Tg mice expressed FcγRII and/or FcγRIII (Fig. 6 *A*, and reference 5). The specificity of staining with mAb 2.4G2 was established by demonstrating that it could be blocked by unconjugated 2.4G2, but not by unconjugated isotype-matched mAb (not shown). In nontransgenic mice, the percentage of $FcγRII/III^+$ ($2.4G2^+$) thymocytes decreased rapidly during gestation (Fig. 6 *A*, and reference 5). In contrast, the percentage of $FcγRII/III^+$ thymocytes remained high in γ551Tg mice throughout fetal development (Fig. 6 *A*). Because total cellularity was reduced in γ551Tg mice (Fig. 4), the total number of $FcγRII/III^+$ thymocytes was nevertheless equivalent to that of controls. Moreover, $FcγRII/III^+$ thymocytes from fetal day 17.5 control and γ551Tg mice appeared phenotypically similar (i.e., $Thy\text{-}1^+CD4^-CD8^-CD3^-HSA^+$; data not shown). These findings suggested that the high percentage of $FcγRII/III^+$ thymocytes in γTg fetal mice reflected their failure to progress further in development.

Recent work has shown that loss of FcγRII/III surface expression precedes the differentiation of early $CD3^-CD4^-CD8^-$ thymocytes to the DP stage (i.e., DN $FcγRII/III^+ \rightarrow$DN $FcγRII/III^- \rightarrow$DP $FcγRII/III^-$; [32]). To determine if thymocyte development was blocked at the DN $FcγRII/III^+ \rightarrow$DN $FcγRII/III^-$ transition stage in γ551Tg mice, we examined the surface expression of FcγRII/III on fetal DN thymocytes. In nontransgenic mice, the percentage of DN thymocytes that express FcγRII/III decreased from 61% at fetal day 15.5 to 32% at fetal day 17.5, and those cells that remained positive expressed reduced levels of FcγRII/III (Fig. 6 *B*). Significant numbers of DP thymocytes were first detected in nontransgenic mice on fetal day 17.5 (Fig. 6 *B*). In contrast, during the

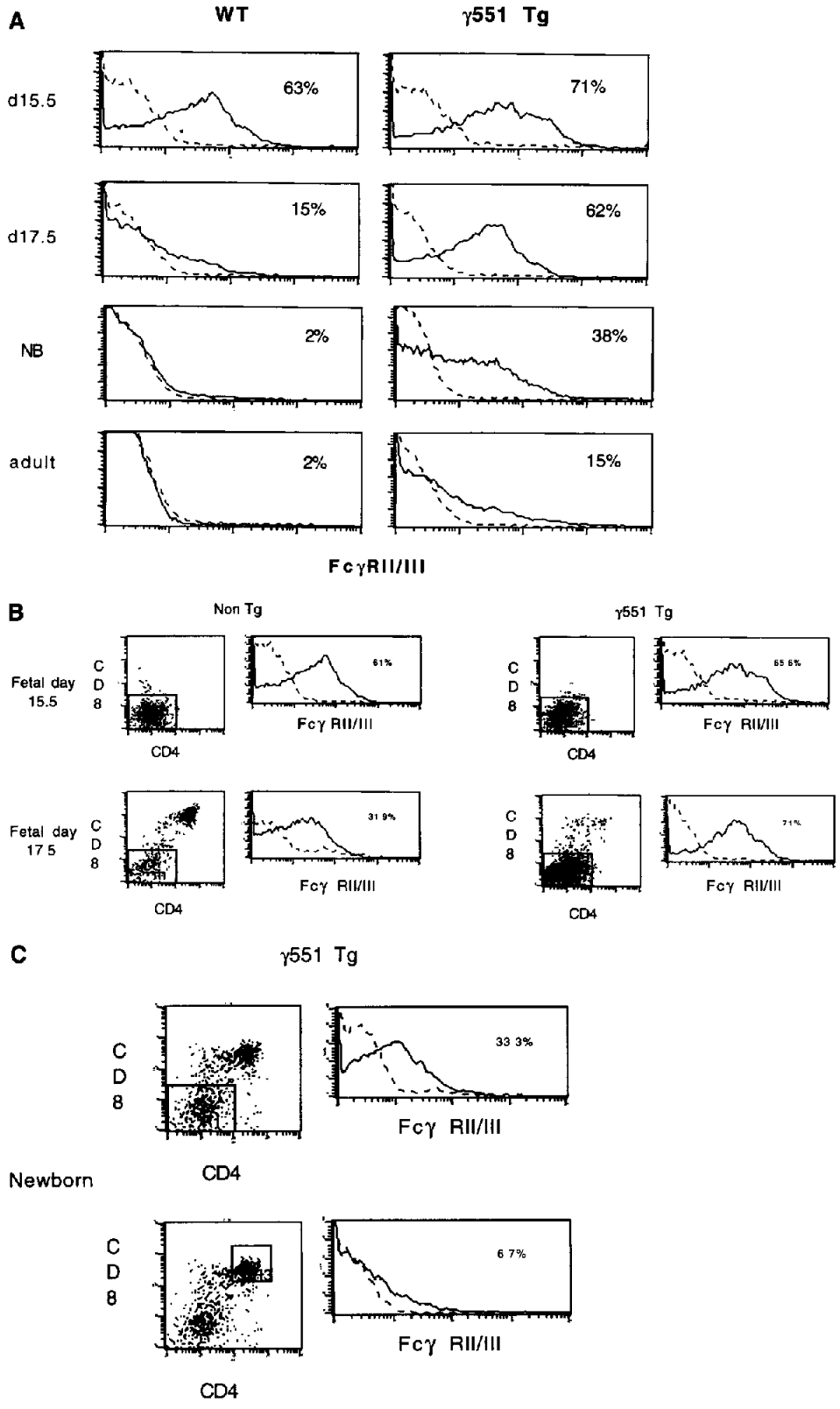

Figure 6. (*A*) Expression of FcγRII and/or FcγRIII on fetal and adult thymocytes from Non Tg and γ551 Tg mice. Total thymocytes were isolated from timed matings of Non Tg and γ551Tg mice at different stages of development (see Materials and Methods). FcγRII/III expression on total thymocytes (*solid lines*) was analyzed by gating on Thy 1.2$^+$ cells. Dotted lines show staining with control antibody, although similar plots were obtained when unlabeled 2 4G2 was added as blocking antibody before staining with conjugated mAb 2.4G2. Numbers in the upper right quadrants indicate the percentage of 2 4G2$^+$ cells. Total 2.4G2$^+$ thymocyte numbers were: fd15.5, Non Tg, 8.6×10^4, γTg, 7.5×10^4; fd17.5, Non Tg, 1.4×10^5, γTg, 2.1×10^5; NB, Non Tg, 2.4×10^5, γTg, 4.8×10^5. (*B*) Analysis of FcγRII/III expression on CD4$^-$CD8$^-$ thymocytes from nontransgenic and γ551 Tg mice. Thymocytes were isolated from pooled fetal day 15.5 or fetal day 17.5 thymi derived from timed matings. Two-color plots show anti-CD4 vs. anti-CD8 staining. For single-color plots, thymocytes were stained with FITC-anti-Thy 1 2, then anti-CD4-PE plus anti-CD8-PE, then biotinylated anti-FcγRII/III (2.4G2) plus avidin-Red 670. Profiles show 2.4G2 staining on Thy-1.2$^+$ CD4$^-$CD8$^-$ thymocytes gated at the time of data collection. Dotted line represents staining of gated cells with control antibody (similar plots were obtained when unconjugated mAb 2 4G2 was added as blocking antibody before staining with biotinylated mAb 2.4G2). (*C*) Analysis of FcγRII/III expression on CD4$^-$CD8$^-$ and CD4$^+$CD8$^+$ thymocytes from newborn γ551Tg mice. Two-color plots show CD4 vs. CD8 staining. Single-color plots show 2.4G2 staining on Thy-1.2$^+$ CD4$^-$CD8$^-$ thymocytes (*left*) or CD4$^+$CD8$^+$ thymocytes (*right*). Thy-1.2$^+$ CD4$^-$CD8$^-$ were analyzed as described in *B*; CD4$^+$CD8$^+$ thymocytes were analyzed by staining with anti-CD4-FITC, then anti-CD8-PE, then biotinylated 2.4G2 plus avidin-Red 670 Gating on CD4$^+$ CD8$^+$ thymocytes was performed at the time of data collection. Dotted line represents staining with control antibody.

same interval (fetal day 15.5–17.5), DN thymocytes from γTg mice failed to differentiate into DP thymocytes, remained predominantly FcγRII/III$^+$ and continued to express high levels of FcγRII/III (Fig. 6 *B*). Appreciable numbers of DN FcγRII/III$^-$ thymocytes were first detectable in newborn γ551Tg mice, indicating that their formation was delayed relative to control mice (Fig. 6 *C*). The appearance of DN FcγRII/III$^-$ thymocytes in newborn

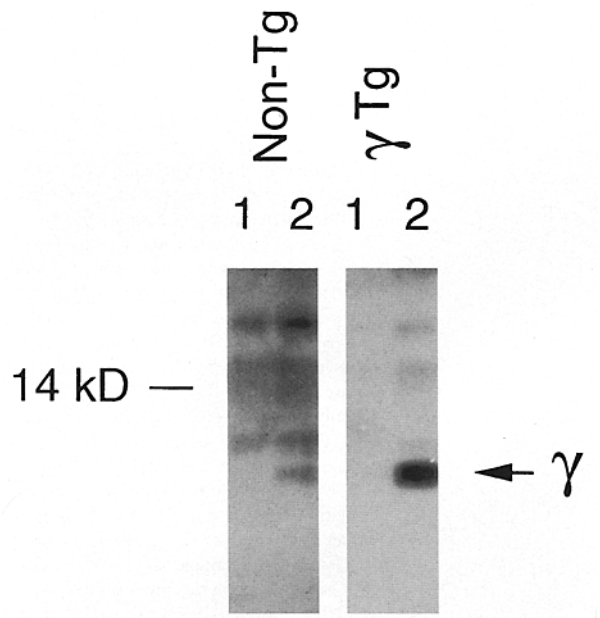

Figure 7. Communoprecipitation of γ chain with mAb 2.4G2. Fetal day 17.5 thymocytes from Non Tg (15×10^6) or γ Tg (8×10^6) mice were lysed in buffer containing 1% digitonin, precleared with protein A–Sepharose plus normal rabbit serum, then incubated with protein A–Sepharose plus rabbit anti–rat antibody (lane *1*), or rabbit anti–rat antibody plus mAb 2.4G2 (lane *2*). Immunoprecipitates were washed, solubilized in reducing SDS loading buffer, and separated by SDS-PAGE (14%). Proteins were transferred to nitrocellulose and blotted with a 1:1,000 dilution of anti-γ sera Immunoreactive proteins were detected by chemiluminescence. Exposure times were 30 s for Non Tg samples and 3 s for γTg samples

γ551Tg mice coincided with the appearance of DP thymocytes, which were also FcγRII/III$^-$ (Fig. 6 *C*). Collectively, these observations indicate that γ overexpression delays the maturation of DN FcγRII/III$^+$ thymocytes.

Expression of FcγRIII in Fetal Thymocytes. mAb 2.4G2 reacts with three distinct FcγR isoforms (RIIb$_1$, RIIb$_2$, and RIII) only one of which (FcγRIII) is known to associate with γ chain. Consequently, we were interested in determining whether the 2.4G2 staining on fetal thymocytes from γ551Tg mice reflected expression of FcγRIII. Reverse transcription–PCR analysis demonstrated that transcripts for both FcγRIIb and FcγRIII (5; data not shown) are expressed in both fetal and newborn thymocytes from control and γ551Tg mice. The presence of FcγRIII (α + γ) complexes was then assessed by immunoprecipitation with mAb 2.4G2 followed by immunoblotting with anti-γ chain sera. As shown in Fig. 7, γ chain could be coprecipitated with mAb 2.4G2 from both nonTg and γ551Tg fetal day 17.5 thymocyte lysates. However, the amount of coimmunoprecipitated γ chain was significantly greater in thymocyte lysates from fetal day 17.5 γ551Tg mice.

Impaired NK Cell Development in γTg Mice. In view of recent data indicating that at least some NK cells may originate from early FcγRIII$^+$ thymocyte progenitors (5, 6), we assessed whether NK cell development was compromised in γTg mice. Analysis of splenocytes (Fig. 8 *A*) and PBL (not shown) demonstrated that the number of NK 1.1$^+$ cells was reduced in γ551Tg mice relative to nontransgenic controls. In contrast, B cell numbers were normal or elevated in these mice (Fig. 8 *A*). As with T cells (indicated by staining with anti-Thy 1.2), the reduction in NK1.1$^+$ cells was transgene dose dependent (Fig. 8 *A*). To assess NK activity, nontransgenic and γ551 Tg mice were injected with poly(IC) and spleen cells were assayed for cytotoxicity against the NK-sensitive target cell, YAC-1, and for ADCC. Consistent with the FACS® analysis, NK activity and ADCC were negligible in γ551 Tg mice (Fig. 8 *B*). Taken together, these results demonstrate that the number of NK cells is markedly reduced in γ551 Tg mice.

Discussion

The present study was directed at exploring a possible role for FcεRIγ in thymocyte development. Northern blot analysis of fetal and adult thymocytes demonstrate that γ is expressed in the fetal thymus: expression is highest at early stages (fetal day 13.5–15.5), and decreases progressively with gestational age. This pattern contrasts sharply with that of the structurally and functionally related ζ chain, which is expressed later in development and functions primarily as a signal-transducing subunit of the α/βTCR complex. In view of these results, we generated transgenic mice that overexpress γ, reasoning that a perturbation of thymocyte maturation, if observed, might yield insight into its function in early thymocytes. Analysis of multiple transgenic founder lines revealed that γ overexpression does indeed impair early thymocyte development. The transgene effects are dose related and result in delayed maturation of fetal DN thymocytes, with less severe effects on later stages of development.

Analysis of fetal thymocytes from high copy number γ551Tg mice revealed that γ chain overexpression specifically blocked thymocyte development at the DN CD25$^+$ FcγRII/RIII$^+$→DN CD25$^-$ FcγRII/RIII$^-$ transition stage. As DN FcγRII/RIII$^+$ fetal thymocytes are thought to include common precursors of T lymphocytes and NK cells (5), it is notable that NK cell development was likewise impaired in γTg mice. Further support for the idea that T and NK cells share a common precursor is provided by the observation that perturbation of both T cell and NK cell development was also observed in transgenic mice in which human CD3ε was overexpressed in early thymocytes (33).

One interpretation of our observations is that the γ transgene–induced effects are mediated by FcγRIII (CD16) complexes on early thymocytes. Although the mAb used to assess FcγRII/III surface expression does not distinguish between these isoforms and therefore does not enable specific quantitative evaluation of FcγRIII surface expression, Western blot analysis is consistent with the idea that γ is associated with FcγRIIIα in both nontransgenic and γTg fetal thymocytes. However, fetal day 17.5 thymocytes from γ551Tg mice, which express high surface levels of FcγRII/III, con-

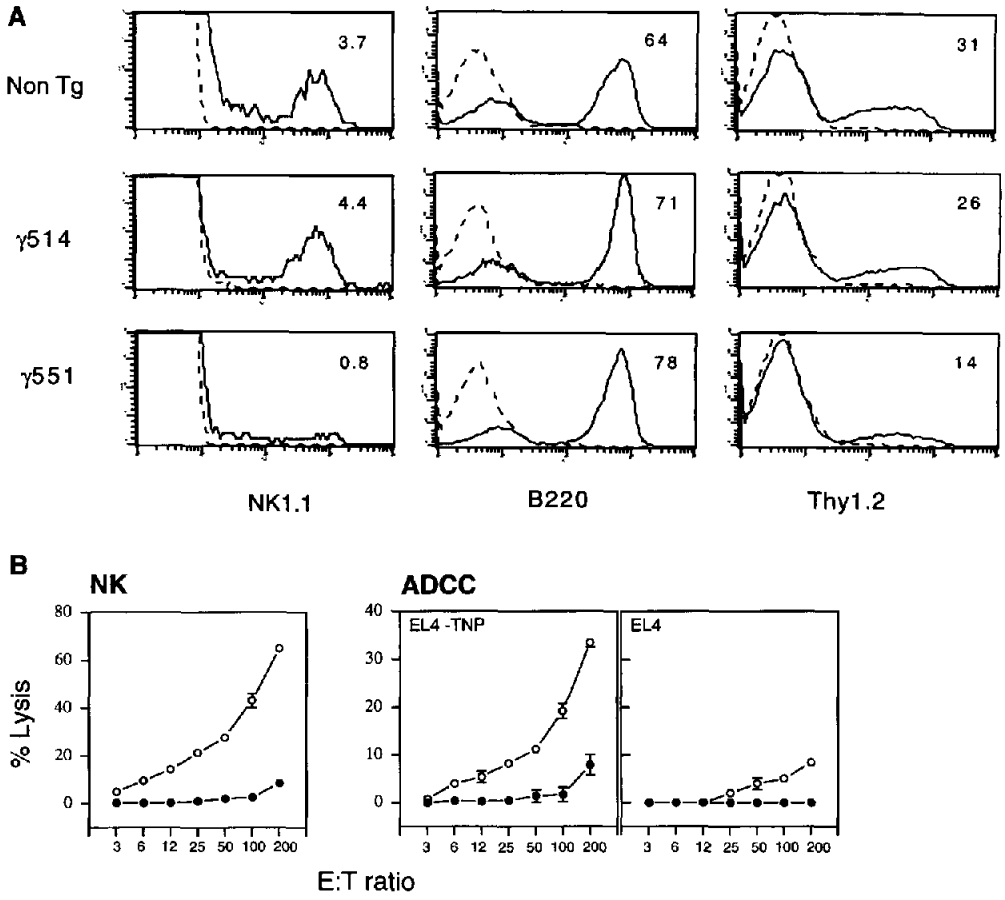

Figure 8. (*A*) Reduction in NK1.1$^+$ cells in γTg mice. Splenocytes from adult nontransgenic (*NonTg*), γ514Tg, and γ551Tg mice were stained with anti-Thy 1.2, anti-B220, or anti-NK1.1, and analyzed by FCM. Single-color plots are shown. Dotted line represents staining with control antibody. Numbers indicate percentage of total. Data are representative of three independent experiments. Total NK1.1$^+$ cells: Non Tg, 3.0×10^6; γ514 Tg, 3.7×10^6; γ551 Tg, 0.6×10^6. (*B*) Reduction in functional NK cells in the periphery of γ Tg mice. NK activity was assessed by cytotoxicity of spleen cells from poly(IC)-treated Non Tg (*open circles*, $n = 2$) or γ551 Tg (*closed circles*, $n = 3$) 10-wk-old C57BL/6 mbred mice against the NK-sensitive target cell line, YAC-1. ADCC was analyzed by determining cytotoxicity of splenocytes (obtained from the same mice used to determine NK activity) against TNP-derivatized EL-4 cells coated with anti-TNP antibody (EL-4-TNP) or non-TNP derivatized EL-4 cells (EL-4). Results shown are the mean ± SEM of triplicate samples.

tained significantly greater amounts of FcγRIII (α + γ) complexes than did age-matched controls, even when absolute numbers of DN thymocytes in each group are taken into consideration. These findings could reflect either an enrichment of DN FcγRIII$^+$ thymocytes in γ551Tg mice, or increased surface expression of FcγRIII on thymocytes from γ551Tg mice. Our own (Fig. 6) and previous data (5, 32) indicate that loss of surface FcγRII/III expression precedes transition of DN thymocytes to the DP stage suggesting that down-regulation of FcγRII/III may be critical for this developmental transition. Thus, increased expression of FcγRIII complexes or failure to down-regulate FcγRIII, might inhibit the further differentiation of early DN thymocytes. Interestingly, the major effects of γ overexpression are observed in early thymocytes as later stages of maturation (that occur after down-regulation of FcγRII/RIII) are less affected.

Seemingly inconsistent with our data is the observation that both T and NK cell development are unaffected in mice rendered γ chain deficient by gene targeting (19). Those results argue that if FcγRIII constitutes a competent signal-transducing complex in early thymocytes, such signals are not essential for thymocyte or NK cell maturation. Although this inconsistency could be explained by functional redundancy, our results favor a hypothesis in which γ chain provides a negative regulatory signal during development. Increased or prolonged signaling by FcγRIII could therefore result in maturational delay, whereas absence of these signals might either appear phenotypically silent or result in accelerated differentiation. In support of this idea is the observation that DN FcγRII/III$^+$ thymocytes are developmentally delayed relative to DN FcγRII/III$^-$ thymocytes (32), and that in fetal thymic organ culture, treatment with mAb 2.4G2, which presumably blocks binding of FcγRII/III to their natural ligand(s), accelerates thymocyte differentiation (20). FcγRIII could function as an early signaling structure expressed on thymocytes before the rearrangement and expression of the clonotypic α/βTCR complex. Triggering of FcγRIII might occur either by direct interaction with its putative ligand or, as suggested previously (5), by its serving as a coreceptor to couple other surface structures (e.g., Thy-1 or CD2) to intracellular signal transduction pathways.

It is also possible that γ chain overexpression inhibits thymocyte development through indirect effects. FcγRIII overexpression could inhibit thymocyte development through sequestration of developmentally important molecules. For example, sequestration of p56^{lck}, which has been shown to interact with γ chain as part of the FcγRIII complex (34), might result in developmental effects such as those described here. In addition, although γ does not associate directly with FcγRII, surface expression of this receptor may also be increased or its expression prolonged in γTg mice. FcγRII has been shown to act as a negative regulator

of hematopoetic cell activation (35); therefore its failure to be down-regulated in early thymocytes could be inhibitory. Since mAb 2.4G2 does not distinguish between FcγRII and FcγRIII we were unable to discern the relative expression of these receptors in non-Tg and γ Tg mice. However, both we (data not shown) and others (5) have detected FcγRIIb expression during fetal thymocyte development by reverse transcription–PCR. Notwithstanding, two lines of evidence indicate that the developmental effects observed in γTg mice are specific to γ chain. First, we have previously shown that overexpression of both η chain or a truncated form of ζ chain that, like γ, contains only a single ITAM does not inhibit thymocyte maturation (22). Second, reconstitution of ζ-deficient ($\zeta^{-/-}$) mice with γ transgenes revealed that the phenotype of γTg mice is identical regardless of the presence of absence of endogenous ζ chain (data not shown). These results indicate that the developmental effects of γ overexpression are not the result of its association with ζ chain or due to competition with ζ chain for association with other molecules.

The phenotype of γTg and ζTg mice also provides insight into the function of these molecules in developing thymocytes. Although T cell development is adversely affected in both ζ and γ transgenic mice, their phenotypes are strikingly different. γ chain overexpression delays, but does not arrest, thymocyte development at the DN FcγRII/III$^+$ stage, whereas ζ chain overexpression results in a near complete arrest at a later stage of development (22). Fetal thymocytes from ζTg mice but not γTg mice are further distinguished by their inappropriate expression of activation markers and premature down-regulation of Rag1 and Rag2 (22). These data suggest that γ and ζ may couple to different signaling pathways in early thymocytes. One possibility is that γ and ζ associate preferentially with different cell surface structures, e.g., ζ chain along with the CD3 subunits as part of the pre-TCR and α/βTCR complexes, and γ chain with the FcγRIII complex. Indeed, the distinctive patterns of γ and ζ expression during gestation would be consistent with this idea.

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References

1. Teh, S.H., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the specificity of the αβ T cell receptor determine the CD4/CD8 phenotype of cells. *Nature (Lond.).* 335:229–233.
2. Fowlkes, B.J., and D.M. Pardoll. 1989. Molecular and cellular events of T cell development. *Adv. Immunol.* 44:207–265.
3. Reth, M. 1989. Antigen receptor tail clue. *Nature (Lond.).* 338:383–384.
4. Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263–274.
5. Rodewald, H-R., P. Moingeon, J.L. Lucich, C. Dosiou, P. Lopez, and E.L. Reinherz. 1992. A population of early fetal thymocytes expressing FcγRII/III contains precursors of T lymphocytes and natural killer cells. *Cell.* 69:139–150.
6. Sánchez, M.J., M.O. Muench, M.G. Roncarolo, L.L. Lanier, and J.H. Phillips. 1994. Identification of a common T/natural killer cell progenitor in human fetal thymus. *J. Exp. Med.* 180:569–576.
7. Kruisbeek, A.M. 1993. Development of αβ T cells. *Curr. Opin. Immunol.* 5:227–234.
8. Zúñiga-Pflücker, J.C., H. Schwartz, and M.J. Lenardo. 1993. Gene transcription in differentiating immature T cell receptor^{neg} thymocytes resembles antigen-activated mature T cells. *J. Exp. Med.* 178:1139–1149.
9. Lanier, L.L., T.J. Kipps, and J.H. Phillips. 1985. Functional properties of a unique subset of cytotoxic CD3$^+$ T lymphocytes that express Fc receptors for IgG (CD16/Leu-11 antigen). *J. Exp. Med.* 162:2089–2106.
10. Ra, C., M-H. Jouvin, and J-P. Kinet. 1989. Complete structure of the mouse mast cell receptor for IgE (FcεRI) and surface expression of chimeric receptors (rat-mouse-human) on transfected cells. *J. Biol. Chem.* 264:15323–15327.
11. Kurosaki, T., I. Gander, and J.V. Ravetch. 1991. A subunit common to an IgG Fc receptor and the T cell receptor mediates assembly through different interactions. *Proc. Natl. Acad. Sci. USA.* 88:3837–3841.
12. Orloff, D.G., C. Ra, S.J. Frank, R.D. Klausner, and J.-P. Kinet. 1990. Family of disulphide-linked dimers containing the ζ and η chains of the T-cell receptor and the γ chain of Fc receptors. *Nature (Lond.).* 347:189–191.
13. Vivier, E., N. Rochet, J.P. Kochan, D.H. Preski, and S.F. Schlossman. 1991. Structural similarity between Fc receptors and T cell receptors. Expression of the γ subunit of FcεRI in human T cells, natural killer cells and thymocytes. *J. Immunol.* 147:4263–4270.
14. Koyasu, S., L. D'Adamio, A.R.N. Arulanandam, S. Abraham, L.K. Clayton, and E. Reinherz. 1992. T cell receptor complexes containing FcεRIγ homodimers in lieu of CD3ζ and CD3η components: a novel isoform expressed on large granular lymphocytes. *J. Exp. Med.* 175:203–209.
15. Ernst, L.K., A.-M. Duchemin, and C.L. Anderson 1993. Association of the high-affinity receptor for IgG (FcγRI) with the γ subunit of the IgE receptor. *Proc. Natl. Acad. Sci. USA.*

90:6023–6027.

16. Scholl, P.R., and R.S. Geha. 1993. Physical association between the high-affinity IgG receptor (Fc gamma RI) and the gamma subunit of the high-affinity IgE receptor (Fc epsilon RI gamma). *Proc. Natl. Acad. Sci. USA*. 90:8847–8850.
17. Qian, D., A. Sperling, D.W. Lancki, Y. Tatsumi, T.A. Barrett, J.A. Bluestone, and F.W. Fitch. 1993. The γ chain of the high-affinity receptor for IgE is a major functional subunit of the T-cell antigen receptor complex in γδ T lymphocytes. *Proc. Natl. Acad. Sci. USA*. 90:11875–11879.
18. Küster, H., H. Thompson, and J.-P. Kinet. 1990. Characterization and expression of the gene for the human Fc receptor γ subunit. Definition of a new gene family. *J. Biol. Chem.* 265:6448–6452.
19. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J.V. Ravetch. 1994. FcRγ chain deletion results in pleiotropic effector cell defects. *Cell*. 76:519–529.
20. Sandor, M., J. Galon, L. Takacs, Y. Tatsumi, A. L. Mueller, C. Sautes, and R.G. Lynch. 1994. An alternative Fcγ-receptor ligand: potential role in T cell development. *Proc. Natl. Acad. Sci. (USA)*. 91:12857–12861.
21. Greaves, D.R., F.D. Wilson, G. Lang, and D. Kioussis. 1989. Human CD2 3′-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell*. 56:979–986.
22. Love, P.E., E.W. Shores, E.J. Lee, A. Grinberg, T.I. Munitz, H. Westphal, and A. Singer. 1994. Differential effects of ζ and η transgenes on early α/β T cell development. *J. Exp. Med*. 179:1485–1494.
23. Fink, P.J., L.A. Matis, D.L. McElligott, M. Bookman, and S.M. Hedrick. 1986. Correlations between T-cell specificity and the structure of the antigen receptor. *Nature (Lond.)*. 321: 219–226.
24. Hedrick, S.M., D.I. Cohen, E.A. Nielsen, and M.M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature (Lond.)*. 308:149–153.
25. Letourneur, O., I.C.S. Kennedy, A.T. Brini, J.R. Ortaldo, J.J. O'Shea, and J.-P. Kinet. 1991. Characterization of the family of dimers associated with Fc Receptors (FcεRI and FcγRIII). *J. Immunol*. 147:2652–2656.
26. Liu, C.-P., R. Ueda, J. She, J. Sancho, B. Wang, G. Weddell, J. Loring, C. Kurahara, E.C. Dudley, A. Hayday et al. 1993. Abnormal T cell development in CD3-$\zeta^{-/-}$ mutant mice and identification of a novel T cell population in the intestine. *EMBO (Eur. Mol. Biol. Organ.) J*. 12:4863–4875.
27. Love, P.E., E.W. Shores, M.D. Johnson, M.L. Tremblay, E.J. Lee, A. Grinberg, S.P. Huang, A. Singer, and H. Westphal. 1993. T cell development in mice that lack the ζ chain of the T cell antigen receptor complex. *Science (Wash. DC)*. 261: 918–921.
28. Ohno, H., T. Aoe, S. Taki, D. Kitamura, Y. Ishida, K. Rajewsky, and T. Saito. 1993. Developmental and functional impairment of T cells in mice lacking CD3ζ chains. *EMBO (Eur. Mol. Biol. Organ.) J*. 12:4357–4366.
29. Malissen, M., A. Gillet, B. Rocha, J. Trucy, E.Viver, C. Boyer, F. Köntgen, N. Brun, G. Mazza, E. Spanopoulou et al. 1993. T cell development in mice lacking the CD3-ζ/η gene. *EMBO (Eur. Mol. Biol. Organ.) J*. 12:4347–4355.
30. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today*. 14:547–553.
31. Unkeless, J.C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med*. 150:580–596.
32. Rodewald, H-R., K. Awad, P. Moingeon, L. D'Adamio, D. Rabinowitz, Y. Shinkai, F.W. Alt, and E.L. Reinherz. 1993. FcγRII/III and CD2 expression mark distinct subpopulations of immature $CD4^-CD8^-$ murine thymocytes: in vivo developmental kinetics and T cell receptor β chain rearrangement status. *J. Exp. Med*. 177:1079–1092.
33. Wang, B., C. Biron, J. She, K. Higgins, M.-J. Sunshine, E. Lacy, N. Lonberg, and C. Terhorst. 1994. A block in early T lymphocyte and natural killer cell development in transgenic mice with high-copy numbers of the human CD3*E* gene. *Proc. Natl. Acad. Sci. USA*. 91:9402–9406.
34. Salcedo, T.W., T. Kurosaki, P. Kanakaraj, J.V. Ravetch, and B. Perussia. 1993. Physical and functional association of $p56^{lck}$ with FcγRIIIA (CD16) in natural killer cells. *J. Exp. Med*. 177:1475–1480.
35. D'Ambrosio, D., K.H. Hippen, S.A. Minskoff, I. Mellman, K.A. Siminovitch, G. Pani, and J.C. Cambier. 1995. PTP1C recruitment and activation by FcγRIIB1: a molecular basis for negative signal cooperativity with the B cell antigen receptor. *Science (Wash. DC)*. 268:263–264.