

Novel mouse mutants with primary cellular immunodeficiencies generated by genome-wide mutagenesis

Thilo Jakob, MD,^{a,b,*} Gabriele V. Köllisch, PhD,^b Maike Howaldt, PhD,^c Mayte Bewersdorff, MSc,^b Birgit Rathkolb, PhD,^c Marcel L. Müller, MD,^a Nadja Sandholzer,^c Lars Nitschke, PhD,^d Matthias Schiemann, PhD,^{e,f} Martin Mempel, MD,^b Markus Ollert, MD,^g Antonie Neubauer, PhD,^h Dian A. Soewarto, MSc,ⁱ Elisabeth Kremmer, MD,^j Johannes Ring, MD, PhD,^k Heidrun Behrendt, MD,^b and Heinrich Flaswinkel, PhD^{c,*} Freiburg, Munich, Erlangen, and Neuherberg, Germany

Background: Primary cellular immunodeficiencies are a group of genetic disorders in which 1 or more components of the cellular immune system are lacking or dysfunctional.

Objective: We sought to identify novel mouse mutants that display primary cellular immunodeficiencies.

Methods: Genome-wide N-ethyl-N-nitrosourea mutagenesis was performed in mice, followed by a phenotype screen of immunologic blood parameters.

Results: We identified novel mouse mutants with isolated B-cell deficiency, combined block in early B- and T-cell development, combined T-cell and natural killer cell reduction, and 3 different forms of T-cell deficiencies. One of the mutants, designated $\Delta T3$, displayed a combined phenotype of increased IgE, absence of peripheral T cells, and block in late thymocyte differentiation. In addition, $\Delta T3$ mice were unable to mount specific humoral immune responses. Chromosomal mapping and sequencing of candidate genes revealed a novel point mutation in the kinase domain of the T-cell receptor ζ chain-associated protein kinase (Zap70). In contrast to Zap70-deficient mice, $\Delta T3$ mutants displayed normal Zap70 mRNA and residual Zap70 protein levels. Complementation studies with Zap70-deficient mice confirmed that the point mutation found in Zap70 was causative for the $\Delta T3$ phenotype, including increased IgE plasma levels, a phenotype that has not been associated with altered Zap70 function in the past.

Conclusion: Random genome-wide mutagenesis combined with a phenotype screen can be used to generate novel mouse mutants with primary cellular immunodeficiencies.

Clinical implications: These mutants can reveal novel genotype-phenotype relationships and might provide novel animal models for different forms of immunodeficiency in human subjects. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: N-ethyl-N-nitrosourea mutagenesis, immunodeficiency, Zap70, IgE, rodents

Primary cellular immunodeficiencies encompass a group of disorders in which, based on genetic defects, 1 or more components of the cellular immune system are lacking or dysfunctional, including T, B, and natural killer (NK) lymphocytes, as well as phagocytic cells.^{1,2} Many of these disorders share clinical phenotypes with common features, such as recurrent infections, chronic inflammation, or autoimmunity. Although some of the disorders present with mild clinical phenotypes, others result in severe infections and significant morbidity and mortality.³⁻⁵ The molecular causes of primary cellular immunodeficiencies are diverse, and over the last decade, a large number of mutations have been identified.⁵

From ^athe Allergy Research Group, Department of Dermatology, University Medical Center Freiburg; ^bthe Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM Center for Allergy and Environment, Technische Universität München, Munich; ^cthe Institute of Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians-Universität München, Munich; ^dthe Department of Genetics, University of Erlangen; ^ethe Institute for Medical Microbiology, Immunology, and Hygiene, Technische Universität München, Munich; ^fthe Clinical Cooperation Group "Antigen-Specific Immunotherapy," GSF-National Research Center for Environment and Health, Neuherberg, and Technische Universität München, Munich; ^gthe Clinical Research Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich; ^hthe Institute for Medical Microbiology, Infectious and Epidemic Diseases, Ludwig-Maximilians-Universität München, Munich; ⁱthe Institute of Experimental Genetics, GSF-National Research Center for Environment and Health, Neuherberg; ^jthe GSF-National Research Center for Environment and Health, Institute of Molecular Immunology, Munich; and

^kthe Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich.

*These authors contributed equally to this work.

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Reprint requests: Thilo Jakob, MD, Professor of Allergy and Immunodermatology, Allergy Research Group, University Medical Center Freiburg, Department of Dermatology, Hauptstr. 7, 79104 Freiburg, Germany. E-mail: thilo.jakob@uniklinik-freiburg.de.

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Abbreviations used

B6: C57BL/6Jlco
 C3H: C3HeB/FeJ
 ENU: N-ethyl-N-nitrosourea
 LAT: Linker for activation of T cells
 NK: Natural killer
 PLC: Phospholipase C
 OVA: Ovalbumin
 TCR: T-cell receptor
 WT: Wild-type
 Zap70: T-cell receptor ζ chain-associated protein kinase

Identification of the exact molecular defects is crucial for early diagnosis, development of treatment strategies, and genetic counseling of family members. In addition, assignment of known genes to different forms of primary immunodeficiencies and identification of novel genes that are involved in the pathogenesis can teach us new aspects of how the immune system works.

Despite our growing knowledge, there are still several forms of primary cellular immunodeficiencies in which the genetic basis is not known.^{1,3} Systematic studies on genetic causes in human subjects are often hampered by the low prevalence of these disorders, the small kindred size, and the variable expression of the phenotype.⁶ The successful genetic analysis of inheritable phenotypic variations of the immune system depends on mutations that occur naturally only at low frequency. In model systems, such as the mouse, this frequency can be increased by using different mutagens, such as N-ethyl-N-nitrosourea (ENU). ENU is a potent mutagen that randomly induces point mutations throughout the genome^{7,8} and has been used in mice extensively. As described previously, we are participating in a large-scale ENU mouse mutagenesis program in which mice are mutagenized and offspring are analyzed for inherited phenotypic variations by using a large panel of different parameters.⁹ Within this program, we have selectively analyzed basal blood parameters of the humoral and cellular immune system^{10,11} and identified several independent mouse mutants that display different phenotypes of cellular immunodeficiencies. Using an outcross backcross breeding scheme and subsequent chromosomal mapping, we were able to identify the chromosomal location of the affected region in 5 of 6 mutants. Moreover, sequencing of a candidate gene in a T cell-deficient mutant mouse allowed us to identify the causative mutation as a point mutation in the kinase domain of the T-cell receptor ζ chain-associated protein kinase (Zap70). Zap70 is a nonreceptor tyrosine kinase containing a tandem SH2 domain. It is crucial for T-cell receptor (TCR) signaling¹² and plays a major role in T-cell activation, thymocyte development, NK cell activation, and NK T-cell development.¹³ In patients with Zap70 deficiency, T cells display an activation defect and abnormal thymocyte development that results in the absence of CD8⁺ cells

in the periphery, whereas CD4⁺ cells are normal in number but do not proliferate on TCR stimulation.¹⁴⁻¹⁷ Recently, a single point mutation leading to a W $\rightarrow$ C amino acid exchange located at the beginning of the second SH2 domain of Zap70 was identified as the causative defect in a mouse mutant that displayed autoimmune arthritis.¹⁸ Thus the phenotypic presentation of different mutations in a given gene can be quite different, depending on the location and type of mutation. In this context it is of interest that the novel Zap70 point mutation described by us not only caused a T-cell deficiency but was also associated with increased plasma concentrations of IgE, a phenotype that has not been reported for Zap70 mutations before.

METHODS

Mice

C3HeB/FeJ (C3H) mice were purchased from the Jackson Laboratory (Bar Harbor, Me), and C57BL/6Jlco (B6) mice were purchased from Ifacredo (l'Arbresle, France). Zap70-deficient mouse mutants¹⁹ were provided by V. Tybulewicz (Mill Hill, London, United Kingdom) on a B10.D2 background. Mice were housed under specific pathogen-free conditions.²⁰ The study was conducted under federal and institutional guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria.

Reagents and antibodies

Anti-CD4 (RM4-5), Pan-NK (DX5), anti-CD122 (TM- β 1), anti-CD19 (1D3), anti-CD8a (53-6.7), anti-CD3e (17A2), anti-TCR β -chain (H57-597), and Fc block (2.4G2) were obtained from BD Biosciences (Heidelberg, Germany). The following antibodies were used for immunoblotting: Zap70 (BD Biosciences), linker for activation of T cells (LAT) (Biomol, Hamburg, Germany), phospholipase C (PLC) γ (Santa Cruz, Heidelberg, Germany), goat antimouse immunoglobulin (BioRad, Munich, Germany), and goat antirabbit immunoglobulin (Dianova, Hamburg, Germany).

ENU mutagenesis and breeding of mice

ENU mutagenesis was performed as described previously.⁹ Offspring (F1) were screened for dominant mutations. F1 mice showing no phenotypic deviation from wild-type (WT) C3H mice in any of the screens were mated to C3H female mice to produce G2 mice. Six to eight G2 female mice were subsequently mated to their F1 fathers to produce G3 offspring.

Screening for mouse mutants

Screening for mutants was performed as described previously.^{10,11} In short, isotype-specific sandwich ELISAs for IgM, IgG1, IgG2a, IgA, and IgE were used to detect differences in the basal level of the respective immunoglobulins. In addition, peripheral blood lymphocytes were analyzed by using flow cytometry with different antibody combinations: (1) CD4/CD19/CD8/CD3e, (2) λ/κ /CD5/CD45, (3) DX5/CD122/CD8/CD3e, (4) CD34/CD62L/CD4/CD44, and (5) H2K^b/b7 integrin/PI control/CD11b.

Ovalbumin sensitization

Mice were sensitized by means of repetitive intraperitoneal injections of ovalbumin (OVA; 1 μ g; Sigma, Deisenhofen, Germany; grade VI)/alum (2 mg; Pierce, Bonn, Germany) in PBS on days 0, 14, 28, 48, and 72. Blood samples were taken at the

indicated time points. OVA-specific IgE, IgG1, and IgG2a levels were measured in plasma samples by means of ELISA, as recently described.²¹

Chromosomal mapping

Chromosomal mapping was based on an outcross of homozygous mutant mice (C3H) to B6 followed by a subsequent brother-sister mating (C3H/B6 × C3H/B6). DNA was analyzed from mutant offspring identified by means of flow cytometry. A genome-wide microsatellite marker panel was applied to pooled DNA from 20 mutant mice to determine the chromosomal location (Medigenomix, Martinsried, Germany). Fine mapping was performed with individual DNA by using a panel of microsatellite markers specific for the respective chromosome.

Sequencing of the candidate gene *Zap70*

Dideoxy sequencing of the *Zap70* gene was performed on an ABI 3100 Genetic Analyzer (AME Biosciences, Torøed, Norway) by using either cDNA or genomic tail DNA as template.

Cell sorting

Double-positive CD4⁺CD8⁺ thymocytes were isolated with a MoFlo cell sorter (Dako, Fort Collins, Colo) and used for measurement of calcium release, RT-PCR, or Western blotting.

Quantitative mRNA analysis

Real-time PCR was performed with RNA from sorted CD4⁺CD8⁺ thymocytes by using intron-spanning primers for *Zap70* (forward, CGACTTTGGCCTGTCCAAAG; reverse, CCTC CCACCTTGATAGATCCAG) and hypoxanthine phosphoribosyl transferase (HPRT) (forward, AGCTACTGTAATGATCAGTCAACG; reverse, AGAGGTCCTTTTCACCAGCA). Relative transcript quantities were calculated by using the Ct method, with HPRT as the reference gene amplified from the samples. Values were expressed as fold induction (2- $\Delta\Delta$ Ct).

Cell lysis, gel electrophoresis, and immunoblotting

Thymocyte cell lysates (10 minutes at 4°C in RIPA lysing buffer) were separated in a 10% Bis-Tris gel (Invitrogen, Karlsruhe, Germany) and transferred to a polyvinylidene fluoride membrane (Immobilon P; Millipore, Schwalbach, Germany). Membranes were blocked for 1 hour at room temperature and incubated at 4°C overnight with primary antibodies (anti-Zap70, 0.05 µg/mL; anti-PLC γ , 1 µg/mL; and anti-LAT, 4 µg/mL). After repeated washing, blots were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-mouse (1:5000) or goat antirabbit immunoglobulin (1:10000). Peroxidase activity was detected with enhanced chemiluminescence (Amersham Life Science, Piscataway, NJ).

RESULTS

Identification of ENU mouse mutants with primary cellular immunodeficiencies

Using systematic screening of offspring from previously ENU-mutagenized mice,^{9,10} we were able to establish a panel of mouse mutants with primary cellular immunodeficiency (Table I). The phenotypes encompass selective B-cell deficiency (Δ B), selective T-cell deficiency in various degrees (Δ T1– Δ T3), one line with reduced CD8 cells and a partial loss of NK cells (Δ T/NK), and one line with complete loss of T and B cells (Δ T/B). All mutants followed a recessive pattern of inheritance.

TABLE I. ENU-induced mouse mutants with primary cellular immune deficiencies

Mouse line	CD4 T cells	CD8 T cells	B cells	NK cells	Mapping status
Δ B	+	+	–	+	10.6-cM interval
Δ T/B	–	–	–	+	8.5-cM interval
Δ T/NK	+	(–)*	+	(–)*	Pending
Δ T1	+	(–)*	+	+	1.0-cM interval
Δ T2	+	(–)*	+	+	2.4-cM interval
Δ T3	–	–	+	+	2.1-cM interval

Names given in the table reflect the major phenotype of the individual mouse mutant.

+, Normal cell population; –, lack of cell population; (–)*, substantial residual population.

Mapping has been started, and in 5 of 6 mutants, the chromosome and the respective interval harboring the mutation have been identified (Table I). In one of the mutants, the Δ T3 mutant, mapping identified a chromosomal region that harbored a strong candidate gene that could account for some aspects of the observed phenotype. The Δ T3 phenotype will thus be described in more detail before results of chromosomal mapping and candidate gene sequencing will be presented.

Δ T3 phenotype: Lack of T cells and increased plasma IgE levels

Δ T3 mutants were identified by increased plasma IgE concentrations and an almost complete absence of T cells in peripheral blood. Although total leukocyte and lymphocyte counts were normal, flow cytometric analysis of peripheral blood lymphocytes revealed a dramatic reduction in CD3⁺ T cells (Fig 1, A) in homozygous mice compared with that seen in WT mice. Staining for CD4 and CD8 revealed that the remaining T cells represented both subsets. Despite the reduction of T cells, there was no sign for hypogammaglobulinemia in Δ T3 mice. On the contrary, plasma IgE concentrations were increased up to 100-fold, and plasma IgG1 levels were also increased, although to a lesser extent (Fig 1, B). Flow cytometric analysis of Δ T3 thymocytes showed a pronounced reduction of CD4 or CD8 single-positive cells in the thymus (Fig 1, C, upper panel). TCR- β expression, which goes along with the development from CD4/CD8 double-positive to CD4 or CD8 single-positive thymocytes, was barely detectable in thymocytes from Δ T3 mice (Fig 1, C, lower panel). Together, these data suggested that Δ T3 mutants are characterized by an almost complete block in thymocyte development at the transition from CD4/CD8 double-positive to CD4 or CD8 single-positive thymocytes that might account for the lack of T cells in the periphery. Indeed, when Δ T3 mutants were analyzed for the presence and percentage of T-cell subpopulations in different organs, the dramatic reduction in the number of peripheral T cells was found in all tissues analyzed (see Table E1 in the Online Repository at www.jacionline.org). Residual CD4 cells in the peripheral blood of Δ T3 mutants contained an increased percentage of Foxp3⁺ cells

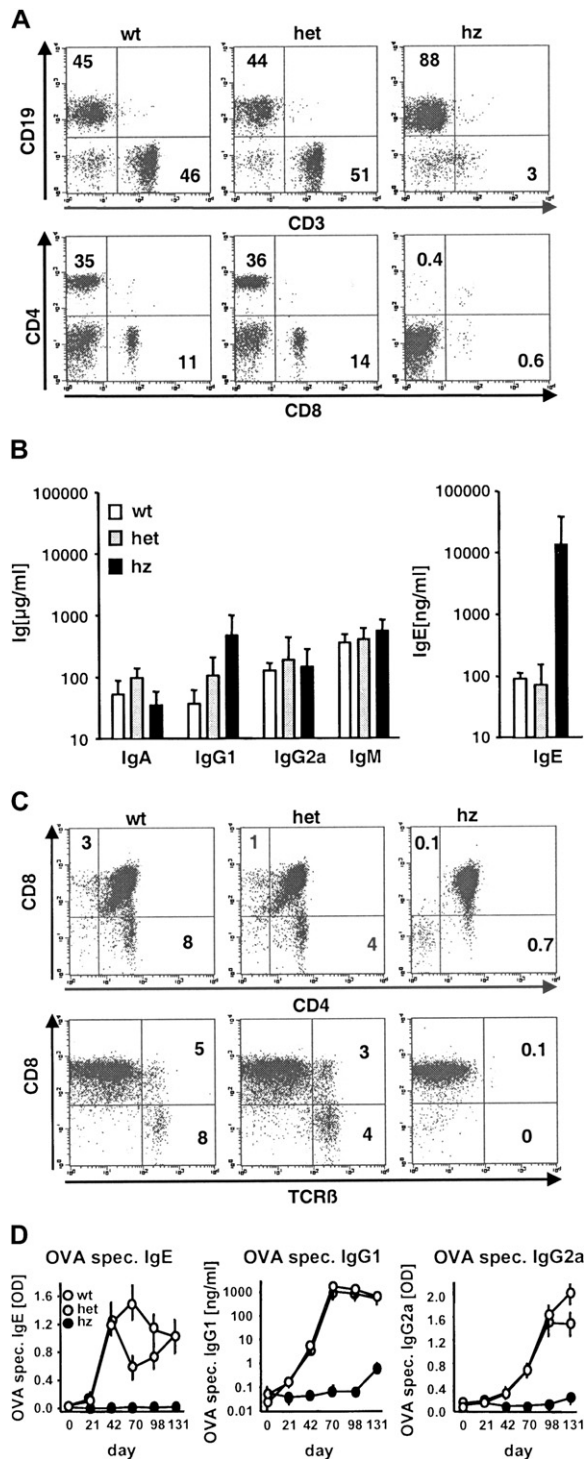


FIG 1. Phenotype of $\Delta T3$ mutants. **A**, Almost complete lack of peripheral T cells in homozygous $\Delta T3$ mutants compared with heterozygous and WT mice (representative results of $n = 10-11$; see Table E1 in this article's Online Repository at www.jacionline.org). **B**, Plasma immunoglobulin concentrations of the indicated isotype in WT ($n = 10$), heterozygous ($n = 10$), and homozygous $\Delta T3$ mice ($n = 11$) at 12 to 14 weeks of age. **C**, Block in thymocyte differentiation at the CD4/CD8 double-positive stage in homozygous $\Delta T3$ mice (representative results of $n = 10-11$; see Table E1 in this article's Online Repository at www.jacionline.org). **D**, OVA-specific

(WT: $3.4\% \pm 0.4\%$, $n = 7$; heterozygous: $4.7\% \pm 0.6\%$, $n = 8$; homozygous: $37.2\% \pm 6.3\%$, $n = 7$; see Fig E1 in the Online Repository at www.jacionline.org). Despite this increase, the absolute numbers of Foxp3⁺ CD4⁺ T cells in peripheral blood were reduced in $\Delta T3$ mutants compared with those seen in heterozygous animals (homozygous: $2.5 \pm 0.8/\mu\text{L}$, $n = 7$; heterozygous: $8.7 \pm 3.2/\mu\text{L}$, $n = 8$). In addition, DX5⁺ TCR- $\alpha\beta$ ⁺ NK T cells were virtually absent in the peripheral blood of homozygous $\Delta T3$ mutants and detectable in lymph node, spleen, and liver leukocyte preparations, albeit at a dramatically reduced level (see Fig E2 in this article's Online Repository at www.jacionline.org). Heterozygous $\Delta T3$ mutants displayed a phenotype that was indistinguishable from that of WT animals in all of the above parameters (Fig 1 and see Table E1 in this article's Online Repository at www.jacionline.org). The *in vivo* functionality of the remaining peripheral T cells was tested by immunizing mice with OVA, a T cell-dependent antigen. In WT and heterozygous mice the immunization resulted in solid antigen-specific IgG1, IgG2a, and IgE responses. In contrast, homozygous $\Delta T3$ mutants were unable to mount specific humoral immune responses (Fig 1, D).

The $\Delta T3$ phenotype maps to chromosome 1 between D1Mit170 and D1Mit172

Analysis of 2 independent pools of DNA from informative C3H/B6 $\times$ C3H/B6 hybrid mouse mutants yielded in both cases a clear association with chromosome 1 (data not shown). Subsequent microsatellite marker analysis with a closely spaced panel of D1Mit markers on individual DNAs detected homozygosity for C3H DNA only in a 2.1-cM interval between D1Mit170 and D1Mit172 (Fig 2, A). This interval comprised 4.7 megabases and harbors 91 genes, of which 22 have a known function.²² One of these genes, *Zap70*, which codes for the src-related nonreceptor tyrosine kinase Zap70, was regarded as a good candidate gene for the observed phenotype.^{19,23} Indeed, sequencing of *Zap70* in $\Delta T3$ mutants revealed a difference in one of the coding exons compared with the published sequence, as well as to WT control mice of the same (C3H) or different genetic backgrounds (BALB/cAnNCrl, C57BL/6-N, FVB/NCrl, DBA/2, JF1, CASA, and SvJ129; data not shown). The mutation is localized at the far C-terminal end of the kinase domain (Fig 2, C). Based on the reading frame, the A to G transition leads to a nonconservative amino acid exchange from glutamic acid (E) to glycine (G) at position 589 (P43404, Universal Protein Resource database, www.ebi.ac.uk).

Expression of Zap70 in $\Delta T3$ mutants

Quantitative mRNA analysis of RNA extracted from sorted CD4/CD8 double-positive thymocytes demonstrated that the single nucleotide exchange had no obvious effect on the steady-state expression level of Zap70

IgE, IgG1, and IgG2a response on OVA/alum sensitization in WT ($n = 7$), heterozygous ($n = 7$), and homozygous ($n = 6$) $\Delta T3$ mice. *het*, Heterozygous; *hz*, homozygous.

A Mapping

Marker	het	het	het	hz	hz	hz	hz	Pos.
D1Mit64	het	het	het	hz	hz	hz	hz	6cM
D1Mit231	hz	het	het	hz	hz	hz	hz	12cM
D1Mit170	hz	hz	het	hz	hz	hz	hz	19.6cM
D1Mit245	hz	hz	hz	hz	hz	hz	hz	20.2cM
D1Mit232	hz	hz	hz	hz	hz	hz	hz	20.8cM
D1Mit321	hz	hz	hz	hz	hz	hz	hz	21cM
D1Mit172	hz	hz	hz	het	hz	hz	hz	21.6cM
D1Mit233	hz	hz	hz	het	hz	hz	hz	23.6cM
D1Mit236	hz	hz	hz	het	het	hz	hz	25.7cM
D1Mit161	hz	hz	hz	het	het	het	hz	27cM
D1Mit130	hz	hz	hz	het	het	het	het	36.9cM

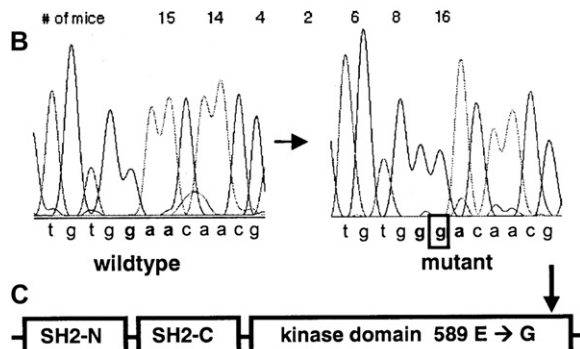


FIG 2. A, Chromosomal mapping reveals a locus defined by the flanking markers D1Mit170 and D1Mit172 (for details, see the Methods section).²² *hz*, Homozygous; *het*, heterozygous. **B**, Sequencing of *Zap70* revealed an A to G transition leading to a nonconservative amino acid exchange at position 589 from glutamic acid (E) to glycine (G). **C**, Location (arrow) of the mutation within the *Zap70* protein at the end of the kinase domain.

mRNA in homozygous $\Delta T3$ mutants compared with heterozygous or WT control animals (Fig 3, A). In contrast, Western blot analysis of cellular extracts from sorted CD4/CD8 double-positive thymocytes revealed a drastic, although not complete, reduction of Zap70 protein in homozygous $\Delta T3$ mutants, whereas expression of LAT and PLC γ , 2 different intracellular adapter molecules associated with TCR signaling, was found to be normal (Fig 3, B). In line with the reduced Zap70 expression, the proliferative response to polyclonal activation by anti-CD3 or concanavalin A (see Fig E3 in the Online Repository at www.jacionline.org), as well as the induction of calcium release through cross-linking of TCRs in double-positive $\Delta T3$ thymocytes, was almost completely abolished (see Fig E4 in the Online Repository at www.jacionline.org).

Zap70-deficient mice do not complement the mutation in $\Delta T3$ mice

Mating homozygous $\Delta T3$ mutants to homozygous Zap70-null mutants generated by means of homologous recombination¹⁹ resulted exclusively in offspring that displayed dramatically reduced percentages of peripheral blood T cells (CD3⁺CD4⁺: 0.25% $\pm$ 0.2%; CD3⁺CD8⁺: 3.0% $\pm$ 1.1%; *n* = 7). Parents, as well as double-heterozygous offspring, show the same phenotype in agreement with a recessive pattern of inheritance. In

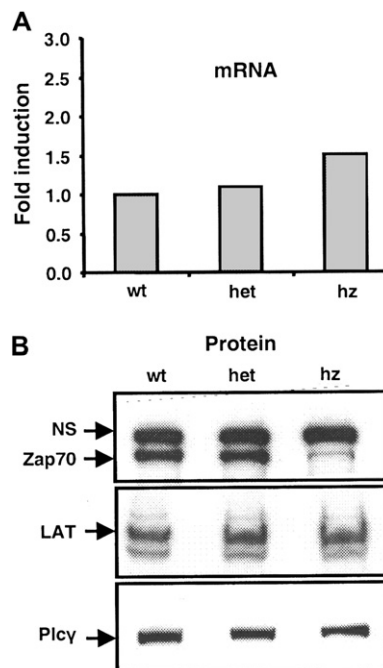


FIG 3. A, Zap70 mRNA from sorted CD4⁺CD8⁺ thymocytes from WT, heterozygous, and homozygous $\Delta T3$ mice (*n* = 2 per experiment), as analyzed by means of quantitative RT-PCR. **B**, Protein expression of Zap70, LAT, and PLC γ from sorted CD4⁺CD8⁺ thymocytes from WT, heterozygous, and homozygous $\Delta T3$ mice, as analyzed by means of Western blotting. *het*, Heterozygous; *hz*, homozygous; NS, nonspecific band.

contrast, mating $\Delta T3$ mutants or Zap70-null mutants with WT mice yielded exclusively normal phenotypes (data not shown). The T-cell deficiency in $\Delta T3$ and Zap70-null mutants was thus caused by the respective mutation of the very same gene. Western blot analysis of Zap70 in these genotypes reflected this pattern. Although Zap70-null mutants were completely deficient of Zap70 protein, $\Delta T3$ and F1 offspring from Zap70-null $\times$ $\Delta T3$ mutants showed residual Zap70 protein (Fig 4, A). In addition to the T-cell deficiency, $\Delta T3$ mutants were characterized by an up to 100-fold increase in basal plasma IgE concentrations. Increased IgE levels had not been reported for Zap70-deficient mice in the past. In line with this, plasma IgE concentrations in Zap70-null mutants were found to be normal (Fig 4, B). When analyzing plasma IgE levels over time, Zap70-null mutants and C3H WT mice showed background-dependent constant low levels of basal IgE (Fig 4, B). In contrast, $\Delta T3$ mutants displayed increased IgE concentrations as early as 10 weeks of age. Interestingly, the double-heterozygous F1 offspring of Zap70-null mutants $\times$ $\Delta T3$ mutants displayed increased plasma IgE concentrations over time (Fig 4, B).

DISCUSSION

Here we describe novel mouse mutants with primary cellular immunodeficiencies that were generated by

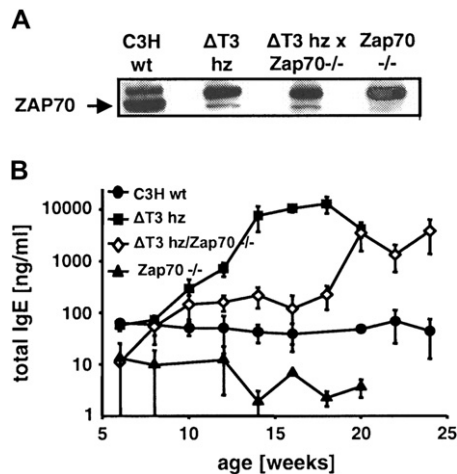


FIG 4. Complementation analysis of $\Delta T3$ mutants and Zap70-deficient mice. **A**, Zap70 Western blot of whole-cell lysates of thymocytes from C3H WT mice, homozygous $\Delta T3$ mutants, Zap70-deficient mice, or F1 offspring of $\Delta T3 \times$ Zap70-deficient mice. **B**, Plasma IgE concentration in WT mice ($n = 4$), homozygous $\Delta T3$ mutants ($n = 7$), Zap70-deficient mice ($n = 7$), and F1 offspring of $\Delta T3 \times$ Zap70-deficient mice ($n = 7$) between 5 and 25 weeks of age. *hz*, Homozygous.

genome-wide ENU mutagenesis and identified by a subsequent phenotype screen of basal blood parameters of the humoral and cellular immune system.⁹⁻¹¹ Subsequent chromosomal mapping allowed us to identify the chromosomal location of the affected region in the majority of the mutants. In one of the mutants, the $\Delta T3$ mutant, the identified chromosomal region harbored a strong candidate gene, *Zap70*, that had been associated with a similar phenotype in the past. Sequencing of *Zap70* identified a point mutation in the kinase domain as the causative mutation for the $\Delta T3$ phenotype.

The majority of alterations observed in $\Delta T3$ mutants closely resemble those observed in mice deficient for Zap70.¹⁹ In contrast to Zap70-deficient mice, $\Delta T3$ mutants displayed normal levels of Zap70 mRNA and residual amounts of Zap70 protein when analyzed by means of Western blotting. To exclude a reduced reactivity of the mutated Zap70 protein with the mAb used for protein analysis, 2 different polyclonal anti-Zap70 antisera raised against different regions of the protein were used instead and confirmed a dramatic reduction of the Zap70 protein level (data not shown). Normal mRNA levels suggested that the reduction of Zap70 protein might be due to problems in translation, instability, and/or increased degradation triggered by the replacement of 1 single amino acid at the end of the kinase domain. At the present time, the exact mechanism leading to the reduced levels of Zap70 protein remains unclear.

The observation that Zap70-deficient mutants do not complement the phenotype of $\Delta T3$ mice demonstrated that the point mutation found in *Zap70* is indeed the causative mutation for the $\Delta T3$ phenotype. Mating of $\Delta T3$ mutants and Zap70-deficient mice resulted in offspring that exclusively displayed the $\Delta T3$ phenotype. This included

the defect in the T-cell compartment, as well as the high levels of IgE. Interestingly, these high levels of IgE are found neither in Zap70-deficient mice nor in mice heterozygous for the $\Delta T3$ phenotype. Moreover, high levels of IgE in offspring of $\Delta T3$ mutants $\times$ Zap70-deficient mice show that 1 $\Delta T3$ allele is sufficient to induce these changes when no WT Zap70 allele is present. Thus even though Zap70 has already been inactivated and spontaneous mutations have been described in the past,^{18,19,24} the novel mutation that we describe in $\Delta T3$ mice implies, for the first time, the direct or indirect involvement of Zap70 in the regulation of IgE.

The dramatic increase in IgE is suggestive of a T_H2 -driven response. Measurement of T_H1 and T_H2 cytokines in peripheral blood T cells was hampered by the scarcity of T cells in the $\Delta T3$ mice. However, when analyzing mitogen-induced IL-4, IL-13, and INF- γ release of thymocytes from WT, heterozygous, and homozygous $\Delta T3$ mice, there was no obvious alteration in cytokine production that would explain an upregulation of IgE production (see Fig E5 in this article's Online Repository at www.jacionline.org). Alternatively, increased IgE levels might result from a lack of inhibitory signals that control IgE production, such as those derived from regulatory T cells. Interestingly, although peripheral blood of $\Delta T3$ mutants contained increased percentages of Foxp3⁺ cells, the absolute numbers in peripheral blood were found to be reduced, which might simply reflect the T-lymphopenic status of the mice. Finally, Zap70 has recently been reported to be expressed throughout B-cell development and seems to play a role in pre-B-cell differentiation and allelic exclusion.²⁵ Thus it cannot be excluded that the E589G mutation in $\Delta T3$ mice might have a direct effect on B-cell development and IgE class switching. Currently, it is not clear whether the phenotype observed in $\Delta T3$ mice might simply reflect the partial loss of Zap70 or whether the E589G mutation leads to structural changes that might result in reduced activation or an impaired kinase activity of Zap70.²⁴ Studies are underway that will address these questions.

A number of differences between murine and human diseases with Zap70 deficiency have been described in the past, and it is important to note that data obtained in mouse models cannot be blindly translated to the immune system in human subjects. However, it also should be noted that the presence of a point mutation in a gene can give rise to a phenotype that is different from the one provoked by its deletion. Although loss of Zap70 leads to a primary cellular immunodeficiency, a single point mutation at the beginning of the second SH2 domain can lead to autoimmune arthritis.¹⁸ The novel point mutation at the end of the kinase described in $\Delta T3$ mice was not associated with autoimmunity, as determined by measurement of anti-DNA antibodies and rheumatoid factor (see Fig E6 in this article's Online Repository at www.jacionline.org) but displayed a profound T-cell deficiency combined with increased IgE production. These results exemplify that we should keep an open mind in seemingly novel primary immune defects and not discard target genes based on a

preset notion of the immune phenotype that has been associated with it in the past.

The other mutants with primary cellular immunodeficiencies listed in Table I are currently at different stages of the respective analysis. The fact that the first identified gene has already been inactivated by homologous recombination is not surprising. Mapping of the respective mutation in a phenotype-driven approach requires excessive resources, effort, and time. A strong candidate, as in the case of $\Delta T3$, speeds up the search for the respective gene dramatically. In contrast, phenotypes caused by novel genes require substantially more meioses to narrow the interval to a region accessible by molecular techniques. It is thus likely that phenotypes caused by hitherto-undefined genes or that resemble novel alleles with gain-of-function properties will unravel their genetic basis and expand our current understanding of the genotype-phenotype relationship in primary cellular immunodeficiencies.

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REFERENCES

- Buckley RH. Primary cellular immunodeficiencies. *J Allergy Clin Immunol* 2002;109:747-57.
- Rosenzweig SD, Holland SM. Phagocyte immunodeficiencies and their infections. *J Allergy Clin Immunol* 2004;113:620-6.
- Notarangelo L, Casanova JL, Conley ME, Chapel H, Fischer A, Puck J, et al. Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee Meeting in Budapest, 2005. *J Allergy Clin Immunol* 2006;117:883-96.
- Bonilla FA, Geha RS. Update on primary immunodeficiency diseases. *J Allergy Clin Immunol* 2006;117(suppl):S435-41.
- Buckley RH. The multiple causes of human SCID. *J Clin Invest* 2004;114:1409-11.
- Casanova JL, Fieschi C, Bustamante J, Reichenbach J, Remus N, von Bemuth H, et al. From idiopathic infectious diseases to novel primary immunodeficiencies. *J Allergy Clin Immunol* 2005;116:426-30.
- Russell WL, Kelly EM, Hunsicker PR, Bangham JW, Maddus SC. Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. *Proc Natl Acad Sci U S A* 1979;76:5818-9.
- Hitotsumachi S, Carpenter DA, Russell WL. Dose repetition increases the mutagenic effectiveness of N-ethyl-N-nitrosourea in mouse spermatogonia. *Proc Natl Acad Sci U S A* 1985;82:6619-21.
- Hrabe de Angelis M, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, et al. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet* 2000;25:444-7.
- Flaswinkel H, Alessandrini F, Rathkolb B, Decker T, Kremmer E, Servatius A, et al. Identification of immunological relevant phenotypes in ENU mutagenized mice. *Mamm Genome* 2000;11:526-7.
- Alessandrini F, Jakob T, Wolf A, Wolf E, Balling R, Hrabe de Angelis M, et al. ENU mouse mutagenesis: generation of mouse mutants with aberrant plasma IgE levels. *Int Arch Allergy Immunol* 2001;124:25-8.
- Chan AC, Iwashima M, Turck CW, Weiss A. Zap70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 1992;71:649-62.
- Hivroz C. Everything you ever wanted to know about Zap70. *Med Sci (Paris)* 2005;21:150-5.
- Chan AC, Kadlec TA, Elder ME, Filipovich AH, Kuo WL, Iwashima M, et al. Zap70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science* 1994;264:1599-601.
- Arpaia E, Shahar M, Dadi H, Cohen A, Roifman CM. Defective T cell receptor signaling and CD8+ thymic selection in humans lacking Zap-70 kinase. *Cell* 1994;76:947-58.
- Hivroz C, Fischer A. Immunodeficiency diseases. Multiple roles for Zap70. *Curr Biol* 1994;4:731-3.
- Elder ME, Lin D, Clever J, Chan AC, Hope TJ, Weiss A, et al. Human severe combined immunodeficiency due to a defect in Zap70, a T cell tyrosine kinase. *Science* 1994;264:1596-9.
- Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, et al. Altered thymic T cell selection due to a mutation of the Zap70 gene causes autoimmune arthritis in mice. *Nature* 2003;426:454-60.
- Kadlec TA, van Oers NS, Lefrancois L, Olson S, Finlay D, Chu DH, et al. Differential requirements for Zap70 in TCR signaling and T cell development. *J Immunol* 1998;161:4688-94.
- Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, et al. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. *Lab Anim* 2002;36:20-42.
- Alessandrini F, Schulz H, Takenaka S, Lentner B, Karg E, Behrendt H, et al. Effects of ultrafine carbon particle inhalation on allergic inflammation of the lung. *J Allergy Clin Immunol* 2006;117:824-30.
- Eppig JT, Bult CJ, Kadin JA, Richardson JE, Blake JA, Anagnostopoulos A, et al. The Mouse Genome Database (MGD): from genes to mice—a community resource for mouse biology. *Nucleic Acids Res* 2005;33:D471-5.
- Negishi I, Motoyama N, Nakayama K, Senju S, Hatakeyama S, Zhang Q, et al. Essential role for Zap70 in both positive and negative selection of thymocytes. *Nature* 1995;376:435-8.
- Wiest DL, Ashe JM, Howcroft TK, Lee HM, Kemper DM, Negishi I, et al. A spontaneously arising mutation in the DLAARN motif of murine Zap70 abrogates kinase activity and arrests thymocyte development. *Immunity* 1997;6:663-71.
- Schweighoffer E, Vanes L, Mathiot A, Nakamura T, Tybulewicz VL. Unexpected requirement for Zap70 in pre-B cell development and allelic exclusion. *Immunity* 2003;18:523-33.

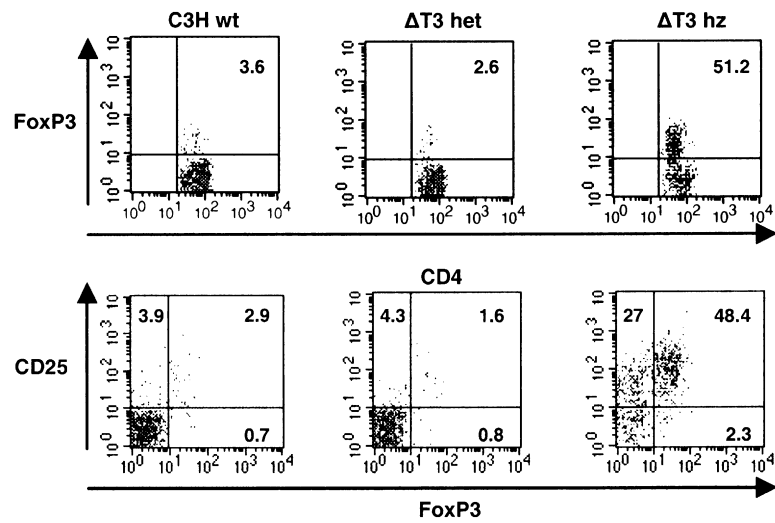


FIG E1. Foxp3⁺ CD4⁺ T cells (*upper panel*) and CD25⁺/Foxp3⁺ CD4⁺ T cells were analyzed in WT (n = 7), heterozygous (n = 8), and homozygous (n = 7) $\Delta T3$ mice. Peripheral blood T cells were stained with anti-CD4, anti-CD25, and anti-Foxp3. Representative data of each genotype gated on CD4⁺ T cells are shown. *het*, Heterozygous; *hz*, homozygous.

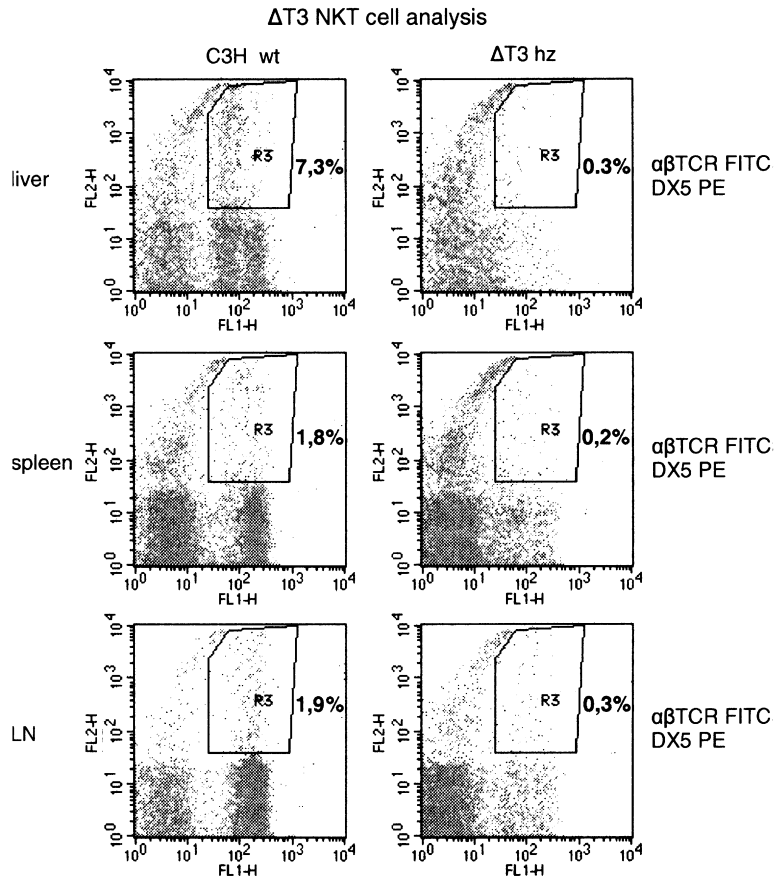


FIG E2. NK T cells were analyzed based on the coexpression of TCR- $\alpha\beta$ and DX5 by means of flow cytometry of cell suspensions from spleen or lymph nodes or leukocyte preparations from liver cell suspension obtained by means of gradient centrifugation. *hz*, Homozygous; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *LN*, lymph node.

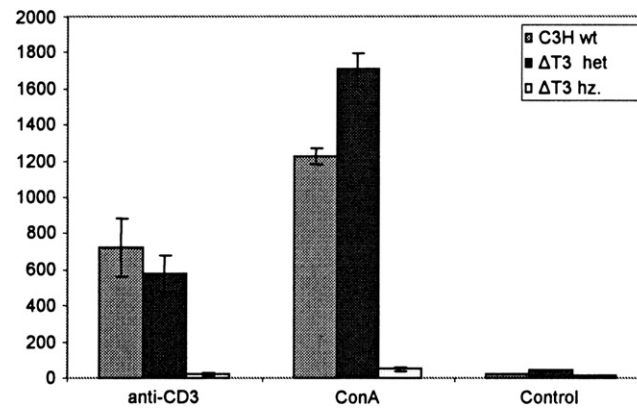


FIG E3. Proliferative response of sorted Thy1.2⁺ T cells from WT mice, ΔT3 heterozygotes, or ΔT3 homozygotes was measured by means of tritiated thymidine incorporation. Sorted Thy1.2⁺ lymphocytes were stimulated with anti-CD3 or concanavalin A for 96 hours, and tritiated thymidine was added for the last 18 hours of culture. *het*, Heterozygous; *hz*, homozygous; *ConA*, concanavalin A.

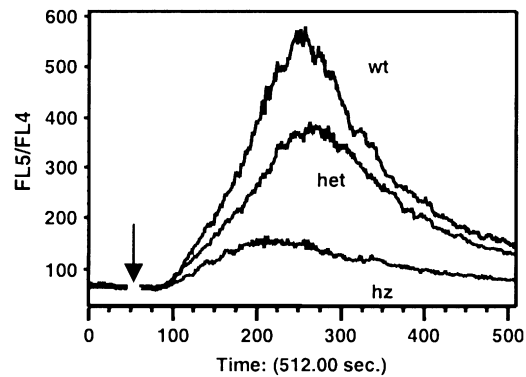


FIG E4. Impaired TCR signaling in $\Delta T3$ mutants. Ca^{2+} release in $\text{CD4}^+\text{CD8}^+$ thymocytes on TCR cross-linking was analyzed by means of flow cytometry, as described below. On TCR cross-linking, homozygous $\Delta T3$ mutants displayed an almost completely blunted Ca^{2+} release, whereas heterozygous mice showed a pronounced but reduced signal compared with WT controls. *het*, Heterozygous; *hz*, homozygous. Thymocytes were prepared as described above, loaded with $4.5\ \mu\text{mol/L}$ Indo-1 (Invitrogen), and stained with fluorochrome-labeled rat antimouse CD4 mAb (RmCD4, Rat IgG2b) and rat antimouse CD8 (RmCD8, Rat IgG2b), and unlabeled anti-TCR mAb (H57, Armenian hamster IgG) on ice. Before stimulation, cells were diluted in prewarmed RPMI 1640 and 1% FCS and analyzed at a concentration of 1×10^6 cells/mL. Once a baseline of fluorescence 5 (FL5)/fluorescence 4 (FL4) had been recorded, cells were stimulated by the addition of $20\ \mu\text{g/mL}$ polyclonal goat anti-hamster IgG (Sigma). Increases in intracellular free Ca^{2+} of double-positive thymocytes were measured as the ratio of FL5/FL4 fluorescence in real time with the use of a FACSvantage (Becton Dickinson) by gating on $\text{CD4}^+\text{CD8}^+$ double-positive thymocytes. Data were processed by using Flow Jo software (TreeStar, San Carlos, Calif).

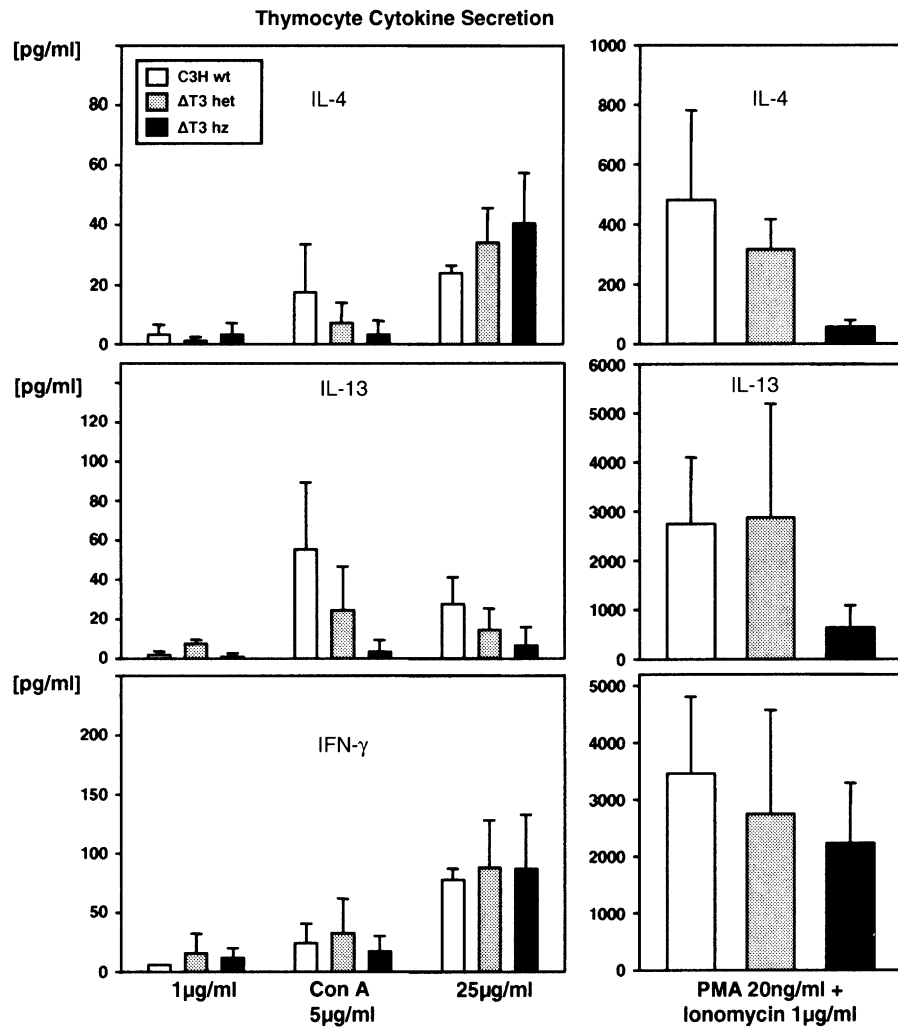


FIG E5. Cytokine production of thymocytes from C3H WT mice (n = 9), ΔT3 heterozygotes (n = 13), and ΔT3 homozygotes (n = 13). Thymocytes were stimulated at 300,000 cells per well with indicated concentrations of concanavalin A or phorbol 12-myristate 13-acetate plus ionomycin, and cell-culture supernatants were collected at 48 hours and analyzed by means of ELISA. *het*, Heterozygous; *hz*, homozygous; *Con A*, concanavalin A; *PMA*, phorbol 12-myristate 13-acetate.

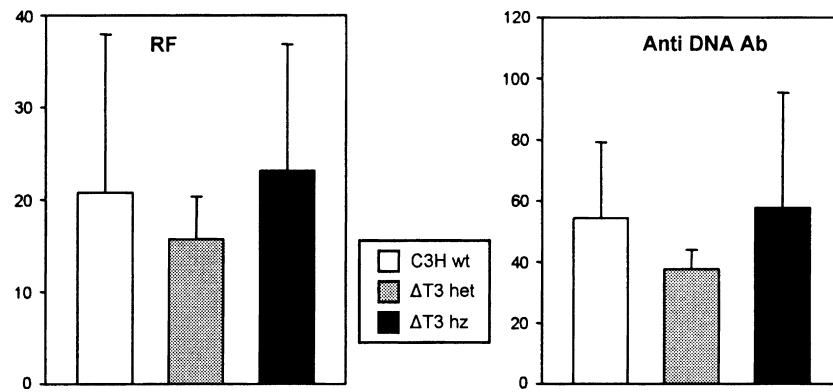


FIG E6. The presence of autoimmunity was assessed by analyzing plasma for the presence of anti-DNA antibodies and RF, as previously described.⁹ RF and anti-DNA antibodies were analyzed in C3H WT mice (n = 21), ΔT3 heterozygous mutants (n = 6), and ΔT3 homozygous mutants (n = 16). Pooled plasma from 6- to 8-month-old *lpr/lpr* mice was used as reference positive control. The results are expressed as arbitrary units normalized to reference plasma set at 800 AU/mL. *RF*, Rheumatoid factor; *het*, heterozygous; *hz*, homozygous; *Ab*, antibody.

TABLE E1. Tissue distribution of T-cell subsets in $\Delta T3$ mice analyzed at the age of 10 to 12 weeks

	Blood				Spleen			
	CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	n	CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	n
WT	46.4 ± 17.1	29.5 ± 12.3	15.9 ± 5.0	10	51.6 ± 11.3	32 ± 71	18.6 ± 5.2	10
het	50.7 ± 12.2	32.3 ± 8.4	17.3 ± 4.1	11	51.1 ± 3.3	31.9 ± 4.7	18.3 ± 3.2	11
hz	5 ± 2.6	3.4 ± 2.4	1.2 ± 1.1	11	7.3 ± 4.2	5 ± 2.9	4.2 ± 3.7	11
	Lymph node				Thymus			
	CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	n	CD4 ⁺ CD8 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	n
WT	55.8 ± 14.0	33.5 ± 13.7	17.3 ± 6.0	6	78.0 ± 4.1	15.4 ± 5.2	3.6 ± 1.2	10
het	62.6 ± 6.2	45.0 ± 3.4	15.6 ± 3.7	6	69.1 ± 10.1	15.1 ± 8.0	4.6 ± 3.1	11
hz	12.8 ± 3.5	6.9 ± 3.5	3.0 ± 1.6	5	85.0 ± 1.4	2.9 ± 0.9	0.3 ± 0.4	11

het, Heterozygous; *hz*, homozygous.