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# Opposing Functions of the T Cell Receptor Kinase ZAP-70 in Immunity and Tolerance Differentially Titrate in Response to Nucleotide Substitutions

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## SUMMARY

**Null mutations that cripple T cell receptor (TCR) signaling explain rare primary immunodeficiencies, but it is not understood why more common polymorphisms that lead to subtle TCR signaling defects are paradoxically associated with autoimmunity. Here we analyzed how a series of *Zap70* variants with step-wise decreases in TCR signaling impacted upon opposing TCR functions of immunity and tolerance. One *Zap70* variant, *murdock*, moderately decreased TCR signaling and thymic selection without compromising immunological tolerance, whereas a more severe *Zap70* defect, *mrtless*, abolished thymic-positive selection and led to immunodeficiency. Signaling capacities between these two thresholds disproportionately compromised negative selection and Foxp3$^+$ regulatory T cell formation, creating a cellular imbalance between immunogenic and tolerogenic functions that resulted in the excessive production of autoantibodies and immunoglobulin E (IgE). The pleiotropic functions of ZAP-70 and their differential response to graded variation provide a paradigm for understanding the complex outcomes of human genetic variation.**

## INTRODUCTION

Precisely how genetic variation affects our susceptibility to infection, autoimmunity, and allergy is only scarcely understood. The largest sources of human genetic variation are single-nucleotide substitutions that occur approximately every 1000 nucleotides and affect all genes, and copy number differences that affect ~12% of genes (Redon et al., 2006). The chief effect of this variation is quantitative changes in the activity of specific gene products. Although the capacity to produce knockout mice has dramatically accelerated knowledge about the immunological consequences of complete loss of specific gene products, we have remarkably little knowledge of the immunological consequences of genetic variation at intermediate states between "wild-type" and "null," even for pathways that are well defined.

Antigen recognition and T cell activation by the TCR is one of the most well defined pathways of the immune system, having been dissected in mice and humans with loss-of-function alleles in many of the critical components (Kane et al., 2000). Loss of key enzymes in this pathway, such as zeta-chain (TCR)-associated protein kinase (ZAP-70), results in severe immunodeficiency in humans and mice resulting from failure of the TCR to signal T cell maturation in the thymus and T cell activation in the periphery (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994; Negishi et al., 1995; Wiest et al., 1997). By contrast, single-nucleotide substitutions affecting ZAP-70 and other molecules in this pathway have recently been identified that do not abolish T cell differentiation or activation but are associated, paradoxically, with autoimmunity and immunopathology. In mice, an amino acid substitution in the C-terminal SH2 domain of ZAP-70 (W163C: *Zap70$^{skg}$*) that decreases TCR signaling also causes autoimmune arthritis when combined with subclinical fungal infection and other genetic factors (Sakaguchi et al., 2003; Yoshitomi et al., 2005). Similarly, a single amino acid substitution in the protein linker of activated T cells (LAT), altering a tyrosine normally phosphorylated by ZAP-70 (LAT$^{Y136F}$), diminishes TCR signaling yet causes a severe inflammatory and hyper-IgE syndrome

in mice (Aguado et al., 2002; Sommers et al., 2002). In humans, a common nucleotide (*PTPN22*$^{1858T}$) and amino acid (R620W) substitution in a protein tyrosine phosphatase, LYP, acts upon lymphocyte protein tyrosine kinase (LCK) upstream of ZAP-70 to decrease TCR signaling (Vang et al., 2005) and has been widely associated with rheumatoid arthritis, Type 1 diabetes, systemic lupus erythematosus, and autoimmune thyroid disease (Begovich et al., 2004; Bottini et al., 2004; Criswell et al., 2005; Kyogoku et al., 2004; Velaga et al., 2004). Also upstream of ZAP-70 is T cell-specific adaptor (TSAd), required for optimal activation of ZAP-70 by LCK (Marti et al., 2006). A promoter polymorphism in the gene encoding TSAd, *SH2D2A*, is associated with multiple sclerosis and juvenile rheumatoid arthritis (Dai et al., 2001; Smerdel et al., 2004). Many more examples of partial defects in the TCR pathway will no doubt emerge as new tools to resequence genes or detect copy number variation are applied. Using this genetic information will hinge upon understanding how partial defects in the efficiency of TCR signaling impact upon regulation of autoimmunity and immunopathology.

It is not known how inherited defects in TCR signaling translate into outcomes such as autoimmunity, hyper-IgE, and immunopathology in some instances but not others. One hypothesis is that the paradoxical variants create a qualitative biochemical imbalance among parallel signaling pathways within individual T cells. This could cripple inhibitory feedback events to a greater extent than activating events, such that T cell hyper-responsiveness or dysregulation occurs. For example, the LAT$^{Y136F}$ point mutation selectively diminishes docking and activation of phospholipase C gamma while preserving docking sites for many other signaling molecules in the LAT scaffold, diminishing calcium flux but not activation of ERK mitogen-activated protein kinase signaling. Similarly, the amino acid substitution in the SH2 domain of ZAP-70$^{skg}$ diminishes ZAP-70 binding to phosphorylated TCR zeta chain immunoreceptor tyrosine-based activation motifs (ITAMs), potentially increasing the recruitment and activation of parallel signaling molecules. An alternative hypothesis is that simple quantitative differences in TCR signaling in some cases create a qualitative cellular imbalance, resulting from opposing actions of the TCR in different T cells. In the thymus, TCR signaling triggers divergent functions of positive and negative selection, depending upon the strength of self peptide-MHC binding to unique TCRs on each thymocyte (Daniels et al., 2006). Diminished TCR signaling caused by ZAP-70$^{skg}$ or LAT$^{Y136F}$ enzymes causes thymocytes with strongly self-reactive TCRs to H-Y antigen to be positively selected instead of negatively selected (Sakaguchi et al., 2003; Sommers et al., 2005). This quantitative change in the positive versus negative selection threshold might not be expected to result in autoimmunity, however, because it also changes the activation threshold for peripheral T cells such that strongly self-reactive T cells that are positively selected cannot be activated by these antigens in the periphery (Sommers et al., 2005). TCR signaling is also needed for the formation and activity of CD4$^+$Foxp3$^+$ regulatory T cells, and a recent study suggests that deficiency of this inhibitory subset accounts for the immunopathology and hyper-IgE syndrome in LAT$^{Y136F}$ mice (Koonpaew et al., 2006). TCR signaling is also needed to maintain peripheral naive T cells, and when populations of these cells are reduced, availability of IL-7 increases to facilitate activation of self-reactive T cells (Baccala and Theofilopoulos, 2005).

Here we analyze how graded changes in TCR signaling affect immune regulation by taking advantage of two mutant mouse strains, each with partial defects in TCR signaling because of amino acid substitutions within the catalytic site of ZAP-70. One substitution had a modest effect on TCR signaling and thymic development whereas the other severely compromised both, with neither allele resulting in autoimmunity or hyper-IgE in a homozygous state. By contrast, autoimmunity and hyper-IgE arose when these two *Zap70* gene variants were intercrossed, yielding animals with intermediate quantities of TCR signaling that breached critical thresholds for thymic-negative selection and thymic-regulatory T cell formation. Our analyses establish that inherited quantitative variation in TCR signaling results in paradoxical autoimmune and immunocompromised states in some circumstances because of distinct cellular thresholds for opposing pleiotropic actions of the TCR.

## RESULTS

### Identification of the *Zap70* Variant Strain *murdock*

The starting point for this study was a mouse genome-wide screen for *N*-ethyl-*N*-nitrosourea (ENU)-induced single-nucleotide substitutions affecting immune regulation (Nelms and Goodnow, 2001). We identified one variant C57BL/6 strain, *murdock* (*mrd*), with fewer peripheral T cells of the naive CD44$^{lo}$ subset (Figure 1A). This T cell trait was inherited in a recessive Mendelian fashion on the C57BL/6 background and in an F2 intercross with the NOD.*H2*$^k$ strain (Figure 1B) where 39 of 156 individuals (25%) exhibited the *murdock* phenotype. A genome-wide scan of pooled DNA from affected F2 mice linked the trait to *D1Mit212* on chromosome 1, with further typing localizing the mutation to an ~11 Mb interval between *D1Mit410* and *D1Mit245* (Figure 1C). Within this interval, the *Zap70* gene (37.06 Mb) was a prime candidate, considering the arrest of αβ T cell development in *Zap70*-deficient mice (Negishi et al., 1995). Sequencing of *Zap70* cDNA from *mrd* homozygotes revealed a single A to T transversion at base 1207 that was not present in the parental C57BL/6 stock (see Figure S1A available online). The mutation substituted an isoleucine codon for phenylalanine (I367F) within the catalytic kinase domain (Figure S1B) and was predicted to alter the dimensions of the ATP-binding pocket within the catalytic cleft (Figure S1C). For comparison, the W504R *mrtless* mutation (described below) changes a conserved residue in the activation loop within the catalytic site. The *murdock* I367F mutation caused no measurable difference in ZAP-70 protein expression between *Zap70*$^{mrd/mrd}$ and wild-type C57BL/6 thymocytes (Figure 1D). In a

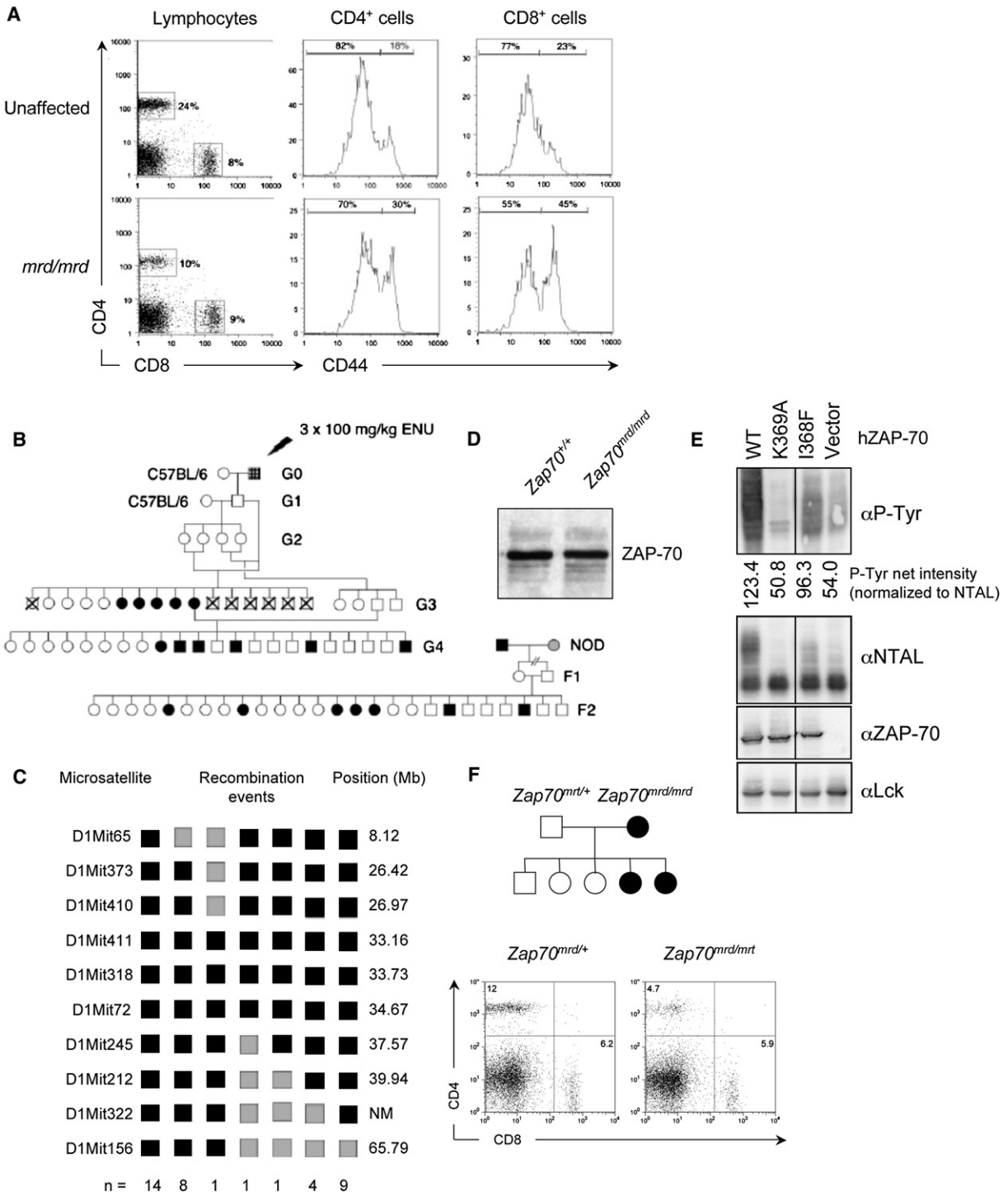



**Figure 1. Fewer Naive T Cells in Mice with a ZAP-70$^{I367F}$ Catalytic Site Substitution, *Zap70$^{mrd/mrd}$***

(A) Representative flow cytometry profiles of CD4 and CD8 expression upon peripheral blood lymphocytes, with representative histograms of CD44 expression on CD4$^+$ and CD8$^+$ T cells from unaffected and *Zap70$^{mrd/mrd}$* mice.

(B) Initial generations of the *Zap70$^{mrd/mrd}$* pedigree, including mapping intercross, showing affected (filled), unaffected (unfilled), and untyped (crossed) mice.

(C) Meiotic mapping of the *Zap70$^{mrd/mrd}$* mutation on chromosome 1. Haplotypes of affected *Zap70$^{mrd/mrd}$* F2IC mice shown in columns: black squares indicate C57BL/6 homozygosity, gray squares indicate C57BL/6-NOD.*H2$^k$* heterozygosity. NM, markers not positioned on the current mouse genome assembly.

(D) Anti-ZAP-70 immunoblot in C57BL/6 and *Zap70$^{mrd/mrd}$* thymocytes.

cotransfection kinase assay, however, the corresponding I368F substitution in human ZAP-70 markedly decreased total (αP-Tyr) and substrate-specific (αNTAL) phosphorylation (Figure 1E).

To confirm that the *Zap70*$^{mrd}$ I367F substitution was responsible for the *murdock* T cell phenotype, we performed a genetic complementation cross with a second mouse strain bearing a mutant copy of *Zap70*, *mrtless* (*mrt*), discovered by the same screen in a parallel pedigree (A.L.Y., O.M.S., S. Lesage, and C.C.G., unpublished data). The *Zap70*$^{mrt}$ allele results from a W504R codon change within the catalytic site, which in the homozygous state reduces ZAP-70 protein expression to 25% of the wild-type quantity and almost completely arrests T cell development at the CD4$^+$CD8$^+$ thymocyte stage. From a *Zap70*$^{mrd/mrd}$ by *Zap70*$^{mrt/+}$ cross, approximately half the offspring exhibited reduced percentages of CD4$^+$ peripheral T cells (Figure 1F), confirming that the *murdock* and *mrtless* T cell traits resulted from noncomplementary alleles. This provided two ENU-induced *Zap70* alleles, with mild and severe losses of function in their respective protein products.

## Spontaneous Production of Autoantibodies and Excessive Secretion of IgE and IgG1 in *Zap70*$^{mrd/mrt}$ Mice

Analysis of antibody production in the compound heterozygous *murdock x mrtless* (*Zap70*$^{mrd/mrt}$) offspring yielded the surprising result that, in contrast to wild-type *Zap70*$^{+/+}$ control mice or the parental *Zap70*$^{mrd/mrd}$ or *Zap70*$^{mrt/mrt}$ strains, 25 out of 27 *murdock x mrtless* mice had IgG autoantibodies reactive to cytoplasmic or nuclear antigens (Figure 2A). Furthermore, *Zap70*$^{mrd/mrt}$ mice spontaneously produced greatly increased amounts of IgE and IgG1 antibodies (Figure 2B). Thus, the inheritance of two different catalytically mutant forms of ZAP-70 triggered dysregulated immune activity in a way that could not be elicited by either mutant form in isolation.

This unique increase in spontaneous antibody secretion in *Zap70*$^{mrd/mrt}$ mice contrasted with diminished acute IgG antibody response to immunization (Figure 2C). Primary Th1 responses to heat-killed *Bordetella pertussis* were particularly sensitive to *Zap70* defects, being equally depressed to ~2% of the wild-type response in each of the three *Zap70* mutant strains. Primary and secondary responses to chicken gammaglobulin (CGG) and secondary responses to Arsonate hapten (ABA) conjugated to CGG were absent in *Zap70*$^{mrt/mrt}$ mice (as might be expected given severe T cell deficiency in these animals) and tended to be lower in *Zap70*$^{mrd/mrt}$ compound heterozygotes than in *Zap70*$^{mrd/mrd}$ animals. Antibody responses to the T-independent antigen NP-Ficoll were normal across all *Zap70* mutant strains. This indicated that *Zap70*$^{mrd/mrt}$ mice had normal B cell activation thresholds and impaired helper T cell function, in spite of enhanced production of autoantibodies and IgE.

## Graded and Threshold Decreases in Helper T Cell Subsets

To understand why autoantibodies, hypergammaglobulinemia, and hyper-IgE were selectively triggered in animals coexpressing two different ZAP-70 catalytic site mutants, we first compared the impact of different *Zap70* genetic states on the number of peripheral CD4$^+$ T cells (Figure 3). As expected, *Zap70*$^{mrt/mrt}$ mice had very few CD4$^+$ T cells. By contrast, *Zap70*$^{mrd/mrd}$ mice had half the number of peripheral CD4$^+$ T cells of wild-type controls (Figures 3A and 3B)—this being due solely to a 70% decrease in naive (CD44$^{lo}$) CD4$^+$ T cells while activated and memory (CD44$^{hi}$) CD4$^+$ T cells remained in normal numbers (Figure 3D). The number of CD44$^{lo}$ CD4$^+$ T cells was decreased by a further 60% in compound heterozygous *Zap70*$^{mrd/mrt}$ animals, whereas there was still no impact on the number of CD44$^{hi}$ cells. Thus, *Zap70* genotype had a graded impact on naive CD4$^+$ T cells in the order *Zap70*$^{mrd/mrd}$ > *Zap70*$^{mrd/mrt}$ > *Zap70*$^{mrt/mrt}$, whereas a distinct threshold was required before the memory and effector compartment was numerically affected. Graded effects on naive CD4$^+$ T cell number were mirrored in TCR-induced calcium responses, which were subtly decreased in T cells homozygous for *Zap70*$^{mrd/mrd}$ but dramatically decreased in *Zap70*$^{mrd/mrt}$ T cells (Figure 3E). A distinct threshold also applied to the CD8$^+$ T cell subset, which was not substantially reduced in *Zap70*$^{mrd/mrd}$ or *Zap70*$^{mrd/mrt}$ individuals (because of a contraction in CD44$^{lo}$ cells and expansion of the CD44$^{hi}$ subset) but was greatly decreased in *Zap70*$^{mrt/mrt}$ animals. The preferential effect of the partial *Zap70* defects on naive CD4$^+$ T cells caused a progressively skewed CD8$^+$:CD4$^+$ T ratio that was most extreme in the *Zap70*$^{mrd/mrt}$ combination (Figure 3C). These different thresholds for effects on CD44$^{lo}$ and CD44$^{hi}$ subsets of CD4$^+$ and CD8$^+$ T cells was consistent with the evidence that these subsets vary in their relative dependence on TCR signals for persistence (Boyman et al., 2007; Caserta and Zamoyska, 2007).

To understand the origins of graded decreases in naive CD4$^+$ T cells, we examined the impact of the different *Zap70* states upon thymocyte differentiation (Figure 4). Formation of CD4$^-$CD8$^+$ thymocytes was not measurably affected by the mild *Zap70*$^{mrd}$ allele, only slightly decreased by the *Zap70*$^{mrd/mrt}$ combination, but almost completely abolished by homozygosity for *Zap70*$^{mrt}$. By contrast, five different manifestations of TCR signal strength (Azzam et al., 1998; Naramura et al., 2002; Sosinowski et al., 2001) showed step-wise decreases in the order *Zap70*$^{+/+}$ > *Zap70*$^{mrd/mrd}$ > *Zap70*$^{mrd/mrt}$ > *Zap70*$^{mrt/mrt}$;

(E) ZAP-70 kinase assay in 293T cells cotransfected with NTAL, LCK, and wild-type (WT) or mutant human *ZAP70* constructs (K369A, kinase-inactive; I368F, *Zap70*$^{mrd}$ equivalent mutation; vector, no *ZAP70* transfected). After 20 hr, cells were lysed and whole-cell lysates were immunoblotted with antibodies indicated on right. Net intensity of P-Tyr (×100), as normalized to NTAL in the corresponding sample, is indicated below respective lanes. (F) Pedigree schematic and low number of T cells in compound heterozygous offspring from parents bearing *Zap70*$^{mrd}$ and *Zap70*$^{mrt}$ missense mutations. Filled symbols denote animals with low T cell numbers.

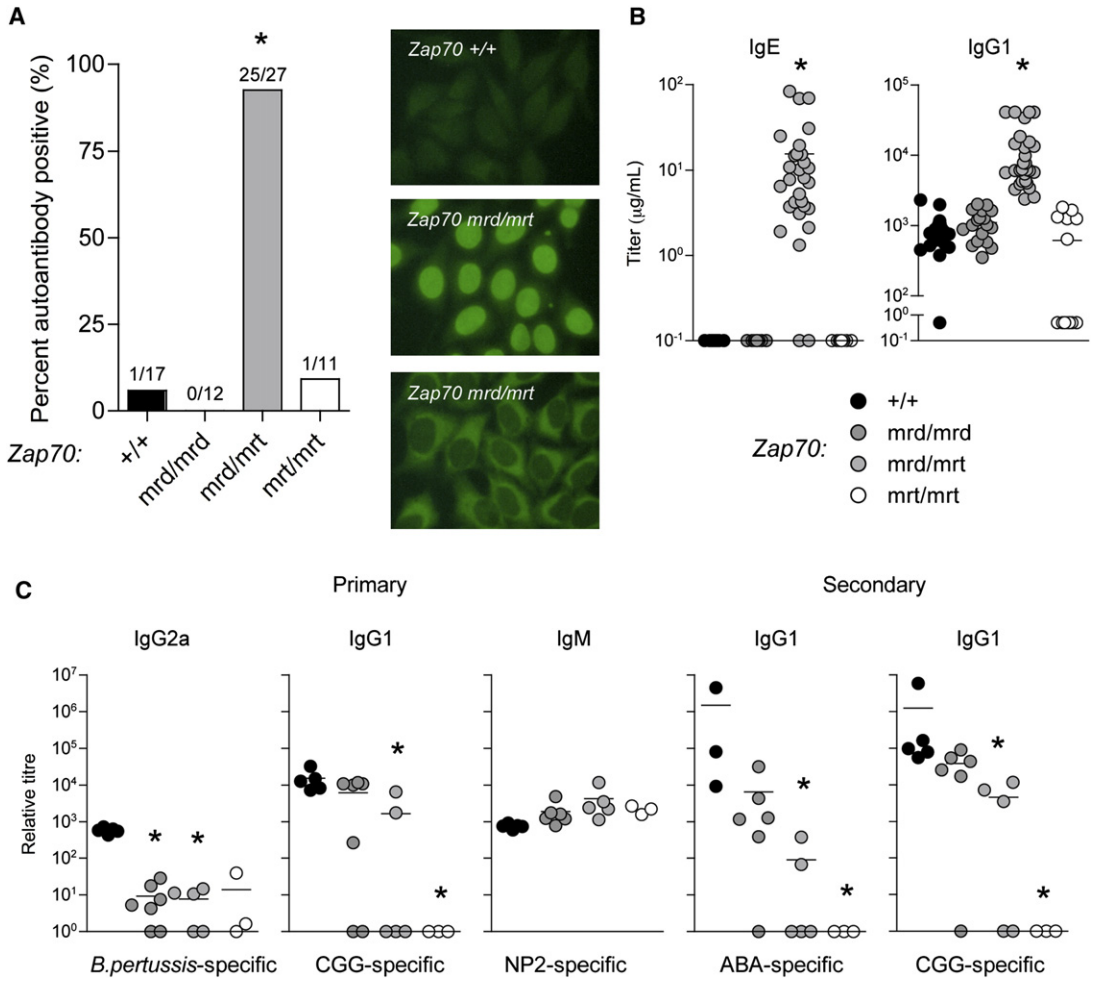



**Figure 2. Autoantibodies, Hypergammaglobulinemia, and Hyper-IgE in *Zap70*$^{mrd/mrt}$, but Not *Zap70*$^{mrd/mrd}$ or *Zap70*$^{mrt/mrt}$ Mice**

(A) Incidence and representative examples of IgG anti-nuclear or anti-cytoplasmic autoantibodies in 8- to 15-week-old mice of the indicated *Zap70* genotypes.

(B) Concentration of serum IgE and IgG1 antibodies in unimmunized mice of the indicated genotypes as measured by ELISA.

(C) Relative ELISA titers of specific antibody to T-dependent (*B. pertussis*, CGG, ABA) and T-independent (NP2) immunogens 14 days and 6 days after primary and secondary immunization, respectively. Asterisk indicates $p < 0.05$.

$CD4^+CD8^-$ T cell numbers (Figures 4A and 4B) and CD5, CD69, TCRβ, and CD3ε expression upon $CD4^+CD8^+$ thymocytes (Figure 4C; Figures S2B and S2C). Incidentally, comparison of CD5 and CD69 expression in *Zap70*$^{mrt/mrt}$ and homozygous null *Zap70*$^{-/-}$ animals established that *Zap70*$^{mrt/mrt}$ thymocytes have greater responsiveness to TCR stimulation than thymocytes with no ZAP-70 (Figure S2B and A.L.Y., O.M.S., S. Lesage, and C.C.G., unpublished data). Decreased TCR and CD3 surface expression also occurred on single positive thymocytes and peripheral T cells in a graded manner with expression on *Zap70*$^{mrd/mrt}$ cells lower than *Zap70*$^{mrd/mrd}$ cells (Figure S2C). Thus, inheritance of the mild *Zap70*$^{mrd}$ allele together with the severe *Zap70*$^{mrt}$ allele diminished TCR signaling to an intermediate point between the parental homozygotes (and intermediate between wild-type and null) with the resulting allelic series representing four distinct efficiencies in TCR signaling.

## Graded and Threshold Defects in Thymic Tolerance in *Zap70*$^{mrd/mrt}$ Mice

To understand why step-wise decreases in TCR signaling and $CD4^+$ T cell formation selectively triggered autoantibodies, hypergammaglobulinemia, and hyper-IgE at only one point in our allelic series, we next asked whether there was a threshold reduction in TCR signaling required to disrupt self-tolerance by clonal deletion. TCR signaling thresholds for negative selection are likely to differ for different TCRs, so we sought a consensus by analyzing polyclonal T cell populations expressing Vβ5 or Vβ11 TCRs with a range of affinities for MMTV self-superantigen complexed with I-E or I-A MHC molecules (Fink et al., 1994; Kappler et al., 1987). In mice with wild-type *Zap70*, a prominent subset of peripheral $CD4^+$ and $CD8^+$ T cells express Vβ5 or Vβ11 in I-E-deficient *H2*$^b$ animals that express only I-A, an MHC molecule that presents the self-superantigen inefficiently (Figure 5A, open circles). These $V\beta5^+$ or $V\beta11^+$

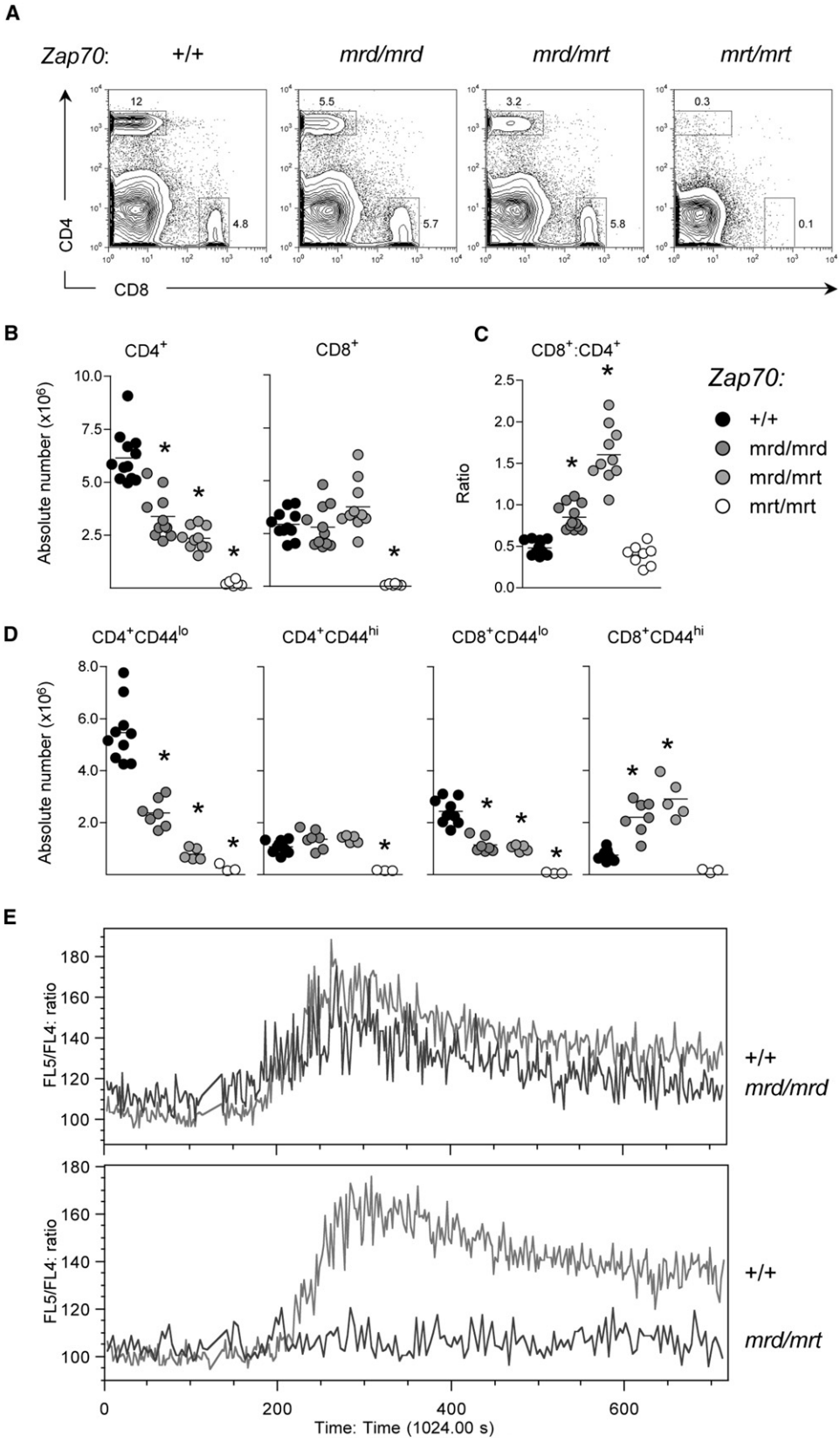
cells are eliminated by thymic-negative selection in $H2^k$ animals expressing I-E, which efficiently presents self-superantigen to the T cells (Figure 5A, filled circles). Homozygosity for the mild $Zap70^{mrd}$ defect interfered with thymic-negative selection in I-E$^+$ $H2^k$ animals, completely precluded negative selection of V$\beta$11$^+$ CD8$^+$ T cells (the least efficiently deleted subset in wild-type mice), and allowed a substantial subset of V$\beta$11$^+$ CD4$^+$ and V$\beta$5$^+$ CD8$^+$ T cells to escape negative selection (Figure 5A). These tolerance events exhibited a low threshold for disruption, as indicated by the fact that they were not further exaggerated in $Zap70^{mrd/mrt}$ compound heterozygotes with a more severe TCR signaling defect. By contrast, clonal deletion of V$\beta$5$^+$ CD4$^+$ T cells by I-E-MMTV exhibited a higher threshold before disruption, because it was mostly intact in $Zap70^{mrd/mrd}$ mice and became defective only in $Zap70^{mrd/mrt}$ animals. Peripheral deletion of V$\beta$5$^+$ CD4$^+$ T cells by I-A-MMTV in $H2^b$ animals (Fink et al., 1994) showed a graded deficit in response to different TCR signaling lesions, with twice as many cells remaining in $Zap70^{mrd/mrd}$ animals and three times as many in $Zap70^{mrd/mrt}$ animals.

To analyze the impact on positive and negative selection of CD4$^+$ T cells bearing a single TCR specificity, $Zap70^{mrd}$ and $Zap70^{mrt}$ alleles were crossed with transgenic mice expressing a hen egg lysozyme (HEL)-I-A$^k$-specific TCR (3A9) and insHEL expressed in pancreatic islet beta cells and thymic medullary epithelium (Akkaraju et al., 1997; Liston et al., 2003). In mice lacking insHEL, the number of CD4$^+$ single-positive thymocytes expressing the 3A9 TCR was diminished to less than 2% of $Zap70^{+/+}$ numbers in the thymus (Figures 5B, 5D, and 5E) and 10% in the spleen (Figure 5C) of animals homozygous for the mild $Zap70^{mrd}$ allele. The small number of clonotype-positive CD4$^+$ T cells that were positively selected in spite of the $Zap70^{mrd}$ mutation may do so either based on signals through the 3A9 receptor or through a coexpressed endogenous receptor. In either case, these data demonstrated that positive selection of some TCRs was acutely sensitive to modest changes in TCR signal strength, thus extending the evidence in Figure 4A that half as many CD4$^+$CD8$^-$ thymocytes matured in $Zap70^{mrd/mrd}$ thymi. Essentially no clonotype-positive CD4$^+$ T cells were present in TCR-transgenic $Zap70^{mrd/mrt}$ compound heterozygotes (not depicted). In the thymus of two sets of TCR insHEL double-transgenic mice analyzed, there was little decrease in the number of clonotype-bearing CD4$^+$ T cells in $Zap70^{mrd/mrd}$ double transgenics compared to $Zap70^{mrd/mrd}$ TCR animals, and their absolute number was higher than the corresponding wild-type double transgenic siblings analyzed at the same time (Figures 5D and 5E). The persistence of clonotype-bright CD4$^+$ SP thymocytes in $Zap70^{mrd/mrd}$ TCR insHEL animals could have either been a direct effect of decreased TCR signaling, an indirect effect of failure to exclude an endogenous TCR-$\alpha$ chain, or a combination of the two. Regardless, these data reinforced the previous evidence that negative selection of some TCRs had a low threshold for disruption by inherited changes in TCR signal strength.

Self-tolerance and suppression of IgG and IgE secretion is also achieved through thymic selection and export of self-reactive CD4$^+$Foxp3$^+$ regulatory T cells (Fontenot and Rudensky, 2005). The absolute number of these cells in the thymus was reduced 4-fold in $Zap70^{mrd/mrd}$ animals (somewhat less than the overall reduction of CD4$^+$ thymocytes) such that they accounted for a slightly increased proportion of CD4$^+$ thymocytes (Figures 6A and 6B). The number of CD4$^+$Foxp3$^+$ T cells was precipitously decreased to 10% of normal numbers in $Zap70^{mrd/mrt}$ thymi—their numbers more severely decreased than CD4$^+$ thymocytes as a whole, such that they comprised a smaller percentage of total CD4$^+$ T cells. In the spleen, however, CD4$^+$Foxp3$^+$ T cells were present in normal numbers in both $Zap70^{mrd/mrd}$ and $Zap70^{mrd/mrt}$ animals, indicating that peripheral homeostasis of this subset was likely to be independent of TCR signaling. As a percentage of splenic CD4$^+$ T cells, Foxp3$^+$ T cells were increased 2-fold because of the selective loss of naive CD4$^+$ T cells in $Zap70^{mrd/mrd}$ and $Zap70^{mrd/mrt}$ mutants. Although a threshold effect upon thymic regulatory T cell formation was masked in the peripheral CD4$^+$Foxp3$^+$ T cell pool, an effect of the $Zap70^{mrd/mrt}$ TCR lesion was nevertheless manifest in peripheral regulatory T cells, which expressed much lower amounts of CD25 compared to those in $Zap70^{mrd/mrd}$ or wild-type mice (Figure 6C). Together, these results demonstrated that in addition to immunogenic mechanisms, tolerogenic mechanisms were also altered by changes in the efficiency of TCR signaling.

## Immune Dysregulation Caused by $Zap70^{mrd/mrt}$ T Cells Is Not Suppressed by Wild-Type T Cells

The more severe defects in CD4$^+$ T cell clonal deletion and in CD4$^+$Foxp3$^+$ regulatory T cells in $Zap70^{mrd/mrt}$ compound heterozygous mice provided two alternative, although nonexclusive, explanations for the selective occurrence of autoantibodies, hypergammaglobulinemia, and hyper-IgE. To test these alternatives, we performed in vivo cell mixing experiments to ask whether or not dysregulated help for antibody secretion was a cell-autonomous trait of $Zap70^{mrd/mrt}$ T cells or if it could be suppressed in the presence of wild-type T cells. First, we injected large numbers ($\sim 3 \times 10^7$) of wild-type spleen cells into young $Zap70^{mrd/mrt}$ mice. Although this did not correct autoantibody or hyper-IgE secretion in $Zap70^{mrd/mrt}$ animals (not depicted), the same procedure corrected a similar antibody dysregulation in a SLP-76 hypomorphic *Lcp2* mutant mouse strain housed under identical conditions (L.A.M., O.M.S., K. Asquith, P. Foster, A.L., and C.C.G., unpublished data). Second, we reconstituted the hematopoietic system of irradiated $Rag1^{-/-}$ mice with mixtures of fetal liver from CD45.1$^+$ $Zap70^{mrd/mrt}$ and wild-type or $Foxp3^{-/Y}$ CD45.2$^+$ donors. All recipients of 100% $Foxp3^{-/Y}$ fetal liver, but not mixtures of $Foxp3^{-/Y}$ and $Zap70^{mrd/mrt}$ liver, became ill 6–7 weeks after reconstitution, indicating a functional competence of $Zap70^{mrd/mrt}$ regulatory T cells sufficient to suppress *scurfy*-like syndrome. In vivo $Zap70^{mrd/mrt}$ regulatory T cell activity was also apparent when $Foxp3^{-/Y}$ splenocytes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Zap70:
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CD4
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12
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Absolute number (x10^6)
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Zap70:
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CD4+CD44lo
CD4+CD44hi
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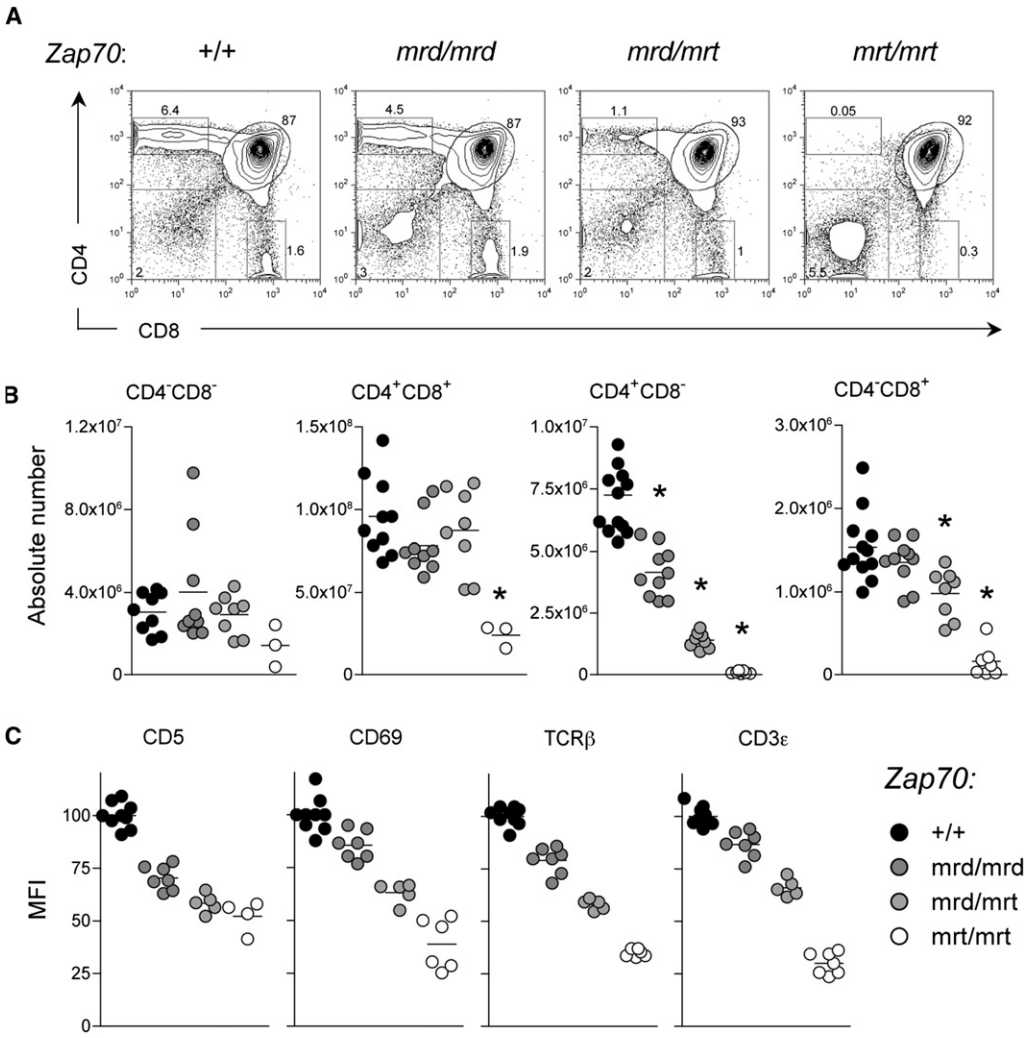


**Figure 4. Four Allelic Graded States of TCR Signaling in Thymocytes**

(A and B) Representative percentage of lymphocytes (A) and absolute number (B) of $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes in B10.Br mice of the indicated *Zap70* genotypes. The lower number of $CD4^+CD8^+$ cells in $Zap70^{mrt/mrt}$ thymi is likely to reflect a difference in age, because it was not significant in other experiments, nor was there any difference in $CD4^+CD8^+$ numbers when compared to $Zap70^{-/-}$ mice (A.L.Y., O.M.S., S. Lesage, and C.C.G., unpublished data).

(C) Normalized mean fluorescent intensities (MFI) of CD5, CD69, TCR-β, and CD3ε expression upon $CD4^+CD8^+$ thymocytes, where the mean value in wild-type cells represents 100 units. Asterisk indicates $p < 0.05$. Representative histograms are shown in Figure S1.

were transferred into $Rag1^{-/-}$ or $Zap70^{mrd/mrt}$ recipients, because $Rag1^{-/-}$ but not $Zap70^{mrd/mrt}$ recipients succumbed to a progressive loss of weight characteristic of inflammatory bowel disease (Figure S2). With respect to serology, a high background of serum autoantibodies in all reconstituted $Rag1^{-/-}$ recipients, regardless of donor cell type, precluded any analysis of the $Zap70^{mrd/mrt}$ contribution to this trait. By contrast, hyper-IgE occurred in all $Zap70^{mrd/mrt}$ reconstituted animals and none of the control animals reconstituted with wild-type fetal liver (Figure 7A). In the mixed chimeras with wild-type or $Foxp3^{-/Y}$ CD45.1 donors, there was no significant decrease in IgE compared to the groups that received only $Zap70^{mrd/mrt}$ fetal liver (Figure 7A). Flow cytometric analysis of the mixed chimeras nevertheless demonstrated that between 10% and 80% of the peripheral $CD4^+$ T cells in these animals were derived from the *Zap70* wild-type $CD45.1^+$ donor (Figure 7B). Moreover, the mixed chimeras displayed

**Figure 3. Graded and Threshold Effects of Different *Zap70* Alleles upon Peripheral T Cell Subsets**

(A–C) Representative percentage of lymphocytes (A), absolute number (B), and ratio (C) of $CD4^+$ and $CD8^+$ T cells in spleens of mice with the indicated *Zap70* genotypes.

(D) Absolute numbers of $CD4^+$ and $CD8^+$ T cells within naive ($CD44^{lo}$) or activated/memory ($CD44^{hi}$) compartments. Asterisk indicates $p < 0.05$.

(E) Anti-CD3-induced calcium flux in $CD4^+$ T cells from wild-type and $Zap70^{mrd/mrd}$ (top) or $Zap70^{mrd/mrt}$ mice (bottom).

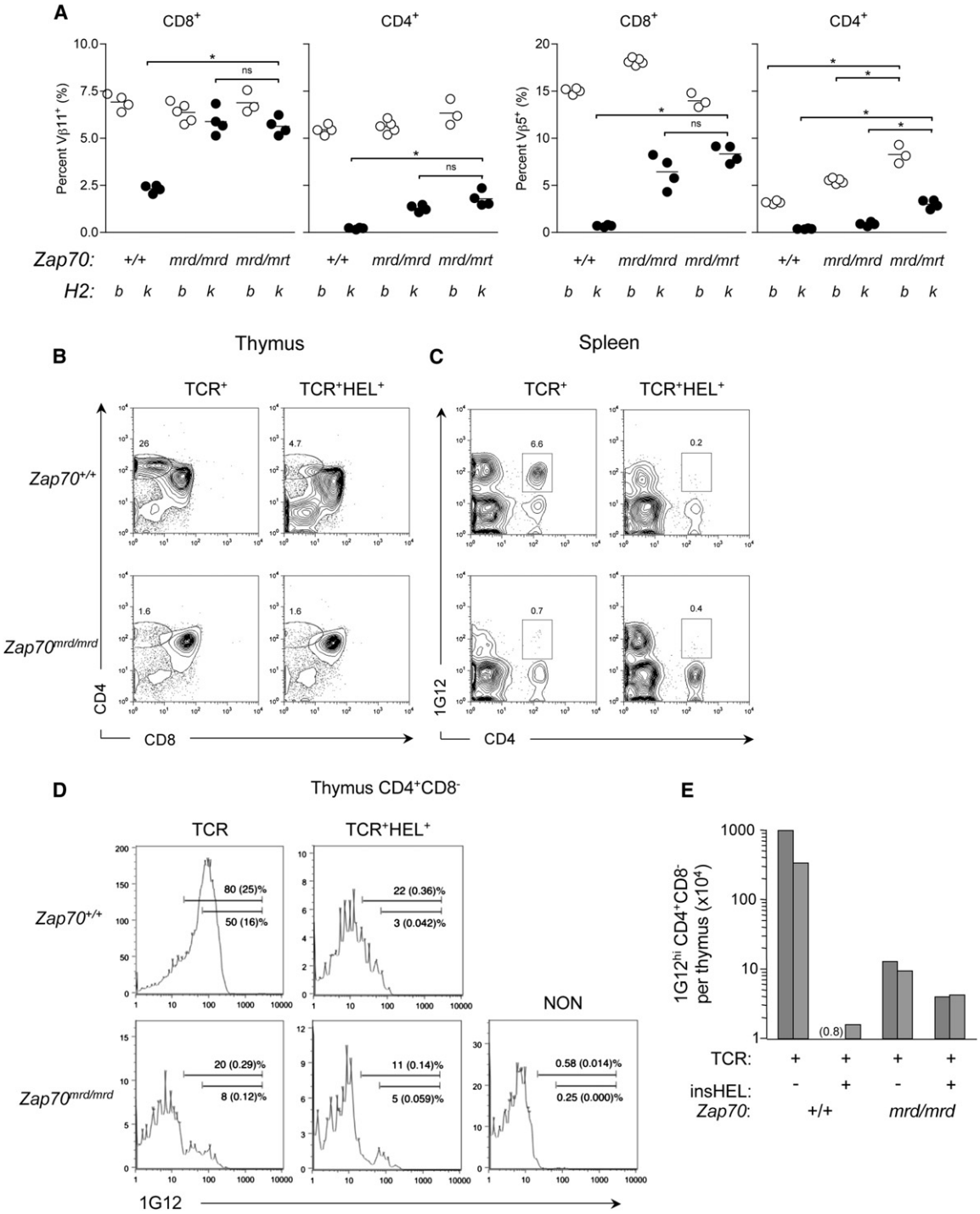


**Figure 5. Threshold Effects of Decreased ZAP-70 Activity on CD4$^+$ T Cell-Negative Selection**

(A) Percentage of CD8$^+$ or CD4$^+$ T cells expressing Vβ11 or Vβ5 in peripheral blood lymphocytes of nontransgenic mice with the indicated *Zap70* genotypes, and either *H2*$^b$ (open symbols) or *H2*$^k$ (filled) haplotypes. Asterisk indicates $p < 0.05$.

(B–D) Representative flow cytometry profiles and (B) percentages of thymocyte CD4$^+$ and CD8$^+$ subsets in TCR transgenic or TCR insHEL double-transgenic mice with wild-type *Zap70* or homozygous *Zap70*$^{mrd/mrd}$.

(C) Spleen cells from the same mice as (B), stained for CD4 and the 3A9 TCR clonotype, 1G12.

(D) 1G12 clonotype staining on gated CD4$^+$CD8$^-$ thymocytes from (B) and from a nontransgenic control. Numbers show percentage of CD4$^+$CD8$^-$ T cells, with bracketed numbers indicating percentage of all thymocytes, that are 1G12$^+$ or 1G12$^{hi}$.

(E) Absolute number of CD4$^+$CD8$^-$ 1G12$^{hi}$ T cells in the thymus of two animals of each genotype.

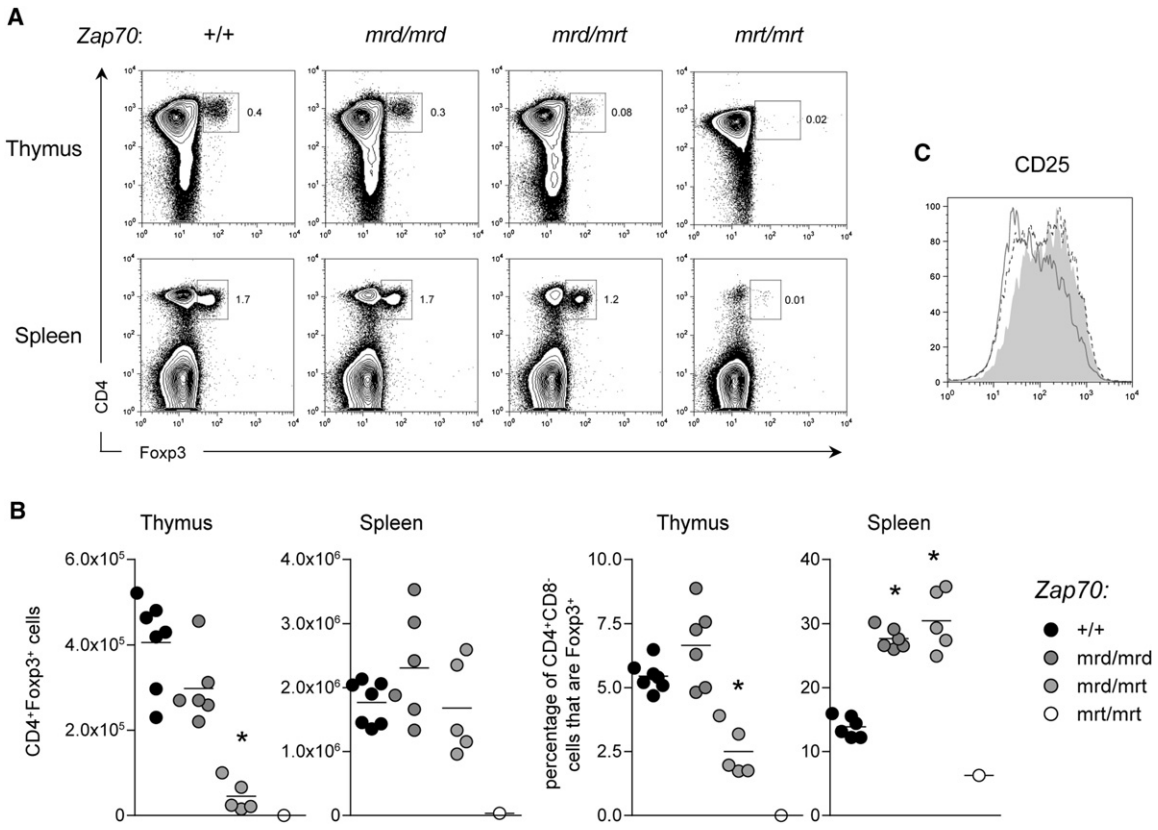


**Figure 6. Threshold Effects of Decreased ZAP-70 Activity on Foxp3$^+$ Regulatory T Cells**

(A) Representative flow cytometry profiles of CD4 and Foxp3 expression and percentage of CD4$^+$Foxp3$^+$ T cells in thymocytes or splenocytes from mice of the indicated *Zap70* genotypes.

(B) Absolute numbers and percentages of CD4$^+$CD8$^-$ thymocytes or splenocytes expressing Foxp3. Asterisk indicates $p < 0.05$.

(C) Representative histogram overlay of CD25 expression upon CD4$^+$Foxp3$^+$ T cells from B10.Br (shaded), *Zap70*$^{mrd/mrd}$ (dotted), or *Zap70*$^{mrd/mrt}$ (solid) mice.

high IgE in spite of the presence of a high proportion of wild-type CD4$^+$Foxp3$^+$ T cells (Figure 7C). Induction of IgE secretion was therefore a cell-autonomous trait of *Zap70*$^{mrd/mrt}$ T cells that was most likely due to the increased frequency of self-reactive CD4$^+$ T cells and could not be complemented by wild-type CD4$^+$Foxp3$^+$ T cells.

## DISCUSSION

Understanding the relationship between common and rare inherited gene variants and immunological disease is a major challenge. Our analysis provides a paradigm for understanding this relationship, by demonstrating how genetic variation resulting in quantitative changes in the activity of a single well-characterized gene and biochemical pathway (*Zap70* and TCR signaling) has pleiotropic threshold effects on the cellular components and overall function of the immune system. By comparing mice inheriting four different allelic states of *Zap70*, we show that system-wide immunological functions and the T cell processes underpinning them have different thresholds of robustness against genetic variation. Least robust were Th1 responses to *Bordetella pertussis* vaccine, thymic CD4$^+$ T cell-positive selection, peripheral naive CD4$^+$ T cell numbers, and thymic-negative selection to trace antigens under Aire control. These failed to varying degrees, even with a subtle *Zap70*$^{mrd/mrd}$ allele. More robust were germinal center memory antibody responses (to ABA hapten), thymic-negative selection of many clones, thymic Foxp3$^+$ regulatory T cell formation, CD25 expression on peripheral Foxp3$^+$ cells, and TCR-induced calcium responses. These nevertheless failed dramatically when a greater genetic perturbation, *Zap70*$^{mrd/mrt}$, was inherited. By contrast, help for secretion of IgE, IgG autoantibodies, and IgG1 antibodies to protein antigens, maintenance of CD44$^{hi}$ memory CD4$^+$ and CD8$^+$ T cells, and thymic-positive selection of CD8$^+$ T cells remained intact in the face of the *Zap70*$^{mrd/mrt}$ combination. This differential sensitivity of help versus tolerance resulted in dysregulated overproduction of IgG and IgE antibodies at intermediate genetic states between wild-type and null. A recognizable primary immunodeficiency syndrome with clear-cut deficiency of naive and memory CD4$^+$ and CD8$^+$ T subsets and antibody response only arises when ZAP-70 is severely compromised in *Zap70*$^{mrt/mrt}$ animals, or when its function is abolished altogether in *Zap70*$^{-/-}$

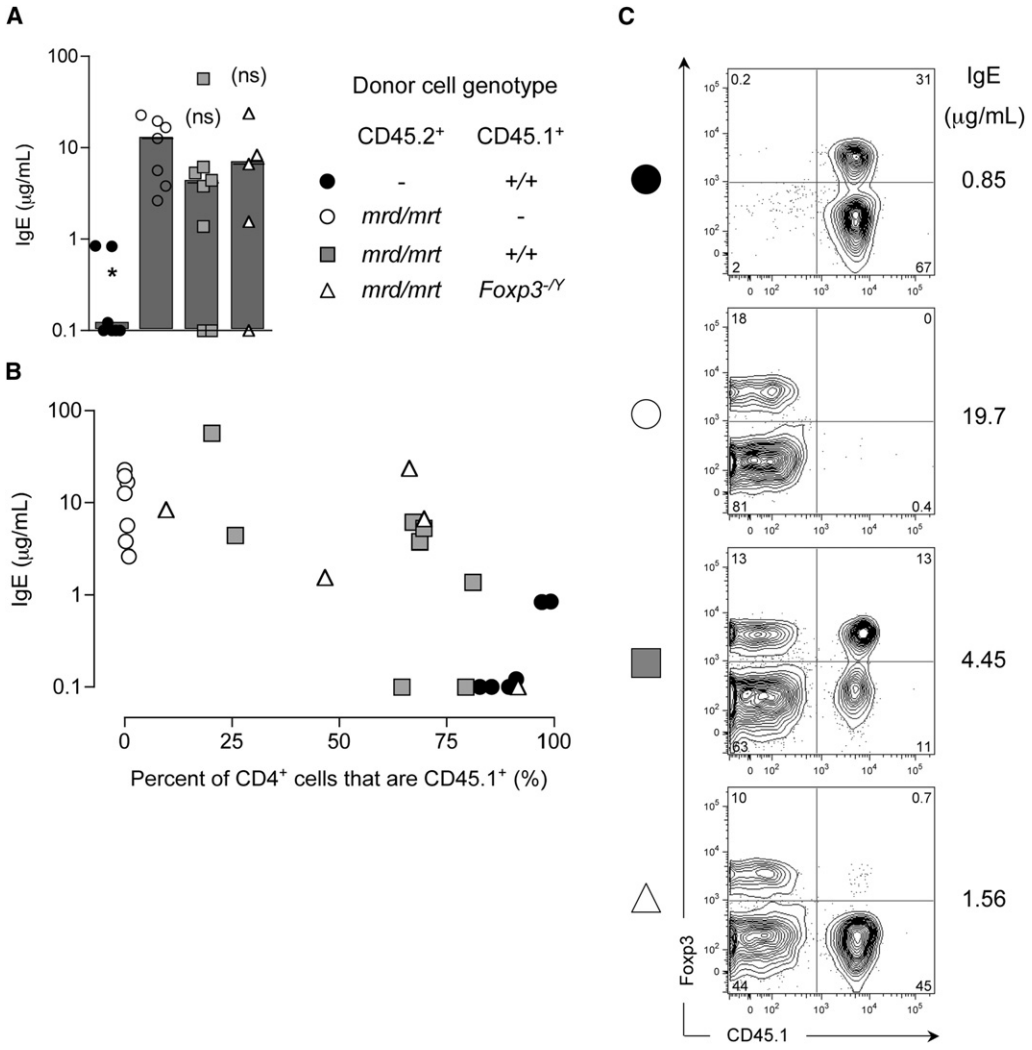


**Figure 7. Failure to Suppress IgE Secretion in *Zap70*$^{mrd/mrt}$ Chimeric Mice with Wild-Type CD4$^+$Foxp3$^+$ T Cells**

(A) Irradiated *Rag1*$^{-/-}$ mice were reconstituted with mixtures of fetal liver cells from CD45.2$^+$ *Zap70*$^{mrd/mrt}$ and CD45.1$^+$ wild-type or *Foxp3*$^{-/Y}$ donors, and serum IgE was measured 6–7 weeks after reconstitution. Columns show median values; ns, no significant difference; asterisk, significant $p < 0.05$, between indicated experimental group and 100% *Zap70*$^{mrd/mrt}$ chimeras by Kruskal-Wallis test with Dunn's post-test.

(B) Chimeric mice were analyzed at the same time for the percentage of CD4$^+$ T cells derived from wild-type CD45.1$^+$ donor stem cells.

(C) Flow cytometry profiles of CD45.1 and Foxp3 expression in CD4$^+$ T cells from representative chimeric recipients. Serum IgE concentrations for each mouse are given alongside.

mice. As discussed below, these findings raise issues about the relative contribution of different genetic and cellular defects to common human immune diseases and their mouse models, and the extent to which genetic and cellular biomarkers may help to stratify common human immune disease.

Autoantibodies, hypergammaglobulinemia, and elevated IgE occur in a number of mouse models where point mutations partially compromise components of the TCR signaling pathway, yet are absent in others. In simplest terms, our analysis of graded defects in ZAP-70 explain these paradoxical outcomes as resulting from lower robustness of regulatory mechanisms compared to helper mechanisms in CD4$^+$ T cells, such that dysregulated help manifests when inherited defects reduce TCR signaling to a critical intermediate point. Considering our *Zap70* series together with other models, the pathogenesis of immune dysregulation in individuals with diminished TCR signaling appears multifactorial and pleiotropic, with variable contributions from defects in thymic deletion, regulatory T cell formation and function, lymphopenia, and immune deficiency and opportunistic infection.

Failure of thymic deletion and autologous responses of CD4$^+$ Th2 or Th17 cells has been suggested to cause antibody dysregulation in LAT$^{Y136F}$ mice (Aguado et al., 2002; Sommers et al., 2005) and arthritis and hypergammaglobulinemia in *Zap70*$^{skg}$ mice (Hirota et al., 2007; Sakaguchi and Sakaguchi, 2005). Nevertheless, this

tolerance defect is insufficient to cause dysregulation in *Zap70*$^{skg}$ mice maintained in a specific pathogen-free (SPF) facility or on a different inbred background (Jiang et al., 2005). Similarly, there was already a marked failure of thymic deletion in *Zap70*$^{mrd/mrd}$ mice that did not translate into autoantibodies or hypergammaglobulinemia, whereas a more severe failure of thymic deletion in *Zap70*$^{mrd/mrt}$ individuals was accompanied by antibody dysregulation. Intuitively, one might expect that a decrease in TCR signaling for thymic deletion would be cancelled out by an equal decrease in TCR signaling for activation in the periphery, such that the system tolerates varying efficiencies of thymic deletion. The fact that hyper-IgE in *Zap70*$^{mrd/mrt}$ mice was dominant in mixed chimeras and was not suppressed by equal numbers of normal T cells or regulatory T cells indicates a T cell-autonomous defect such as escape from thymic deletion. Revealing the signaling pathways and effector mechanisms required for aberrant antibody production, through the introduction of additional mutations, will be a point of future interest.

The role of lymphopenia and regulatory T cell deficiency in immune dysregulation from partial TCR signaling defects also appears to contribute to varying degrees. Elevated IgE is characteristic of regulatory T cell deficiency caused by mouse and human *FOXP3* mutations and of hypomorphic mouse and human *RAG* mutations where there is a limited repertoire of competing T cells (Khiong et al., 2007; Marrella et al., 2007; Villa et al., 1998). Although antibody dysregulation in our *Zap70* allelic series correlated with a marked drop in thymic Foxp3$^+$ CD4$^+$ T cells and decreased CD25 expression on the remaining cells, hyper-IgE in *Zap70*$^{mrd/mrt}$ mice was nevertheless not suppressed by normal T cells or regulatory T cells. This is a point of contrast with LAT$^{Y136F}$ mice, in which pathology can be prevented by the transfer of 2–3 × 10$^5$ CD4$^+$CD25$^+$ T cells (Koonpaew et al., 2006). CD4$^+$CD25$^+$ adoptive T cell transfer has also been reported to prevent arthritis in *Zap70*$^{skg}$ mice (Hirota et al., 2007; Sakaguchi and Sakaguchi, 2005). These differing outcomes may be explained by the robustness of different T cell mechanisms: autoreactive helper cells in *Zap70*$^{mrd/mrt}$ mice may retain sufficient TCR signaling for dysregulated helper function in the face of normal regulatory T cells. Alleles that produce enzymes with further reduced TCR signaling, such as ZAP-70$^{skg}$ or LAT$^{Y136F}$, may weaken autoreactive T helper function to an extent that dysregulated help only occurs when regulatory T cells are also crippled.

In addition to intrinsic T cell dysregulation, it has to be considered that defective effector T cell functions that allow opportunistic infection may play a contributory role. Primary immunodeficiency disorders in man are often associated with apparent autoimmune phenomena that stem from a failure to control viral, bacterial, and fungal infections (Arkwright et al., 2002). Opportunistic, subclinical fungal infection with *Pneumocystis* or *Aspergillus* has been shown to be an essential cofactor in arthritis development in *Zap70*$^{skg}$ mice and appears to act by activating dendritic cells, decreasing T cell IL-4 production, and increasing T cell interferon gamma production and IgG secretion (Kobayashi et al., 2006; Yoshitomi et al., 2005). These events transpire only in *Zap70*$^{skg}$ mice on a susceptible BALB/c background (Jiang et al., 2005). The finding that dysregulated IgE production in *Zap70*$^{mrd/mrt}$ mice was not suppressed by large numbers of normal T cells in adoptive transfer or mixed chimeras would argue against opportunistic infection, because wild-type T cells should restore immune competence. Moreover, Th1 responses to *Bordetella* immunization were equally crippled by the *Zap70*$^{mrd/mrd}$ and *Zap70*$^{mrd/mrt}$ alleles, yet only the latter was associated with dysregulated IgG and IgE secretion. Like the role for regulatory T cell deficiency, there may be variable interplay between primary T cell dysregulation and opportunistic infection in mice with partial TCR signaling lesions, depending upon the extent to which the signaling pathway is compromised and upon other background genes.

A key issue highlighted by our *Zap70* allelic series is the extent to which genetic variation resulting in quantitative changes in components of the TCR signaling pathway blur the lines between immune dysregulation and primary immunodeficiency. Given the number of genes encoding components required for TCR signaling, the substantial germline mutation rate, and large size of the human population, comparable partial defects to *Zap70*$^{mrd/mrt}$ may arise with substantial frequency in our population through additive effects of inheriting heterozygous single-nucleotide or copy-number variant alleles affecting two or more elements of the TCR signaling pathway. The recent discovery of a common functional polymorphism in *PTPN22* associated with various autoimmune diseases is likely to be the tip of the iceberg of sporadic autoimmune or allergic conditions where there is an underlying quantitative defect in TCR signaling that does not meet the extreme criteria for primary immunodeficiency. In the future, biomarker assays based on the least robust T cell mechanisms defined here, combined with resequencing of genes in the TCR pathway, may provide a more sensitive and specific way to stratify common immune dysregulation disorders with this root cause.

## EXPERIMENTAL PROCEDURES

### Mice

*Zap70*$^{mrd/mrd}$ (MGI:3614796) was derived from a C57BL/6 G0 male treated intraperitoneally with 100 mg/kg *N*-ethyl-*N*-nitrosourea (Sigma) at three weekly intervals. Mutation mapping and DNA sequencing were performed as previously described (Miosge et al., 2002). *Zap70*$^{mrd/mrd}$ and *Zap70*$^{mrt/mrt}$ (MGI:3614790) strains were created on a C57BL/6 background and analyzed on a mixed background with B10.Br (B10.BR-H2$^k$/SgSnJ). 3A9 TCR transgenic (Ho et al., 1994) and ILK-3 insHEL transgenic (Akkaraju et al., 1997) mice were produced on the C57BL/6J background, backcrossed more than seven generations to B10.Br, backcrossed to *Zap70*$^{mrd/mrd}$ and *Zap70*$^{mrt/mrt}$ strains, and finally intercrossed. *Rag1*$^{-/-}$, *Foxp3*$^{-/Y}$, NOD.*H2*$^k$, and C57BL/6.Ly5a congenic mice have all been described previously (Fontenot et al., 2003; Lesage et al., 2002; Miosge et al., 2002; Mombaerts et al., 1992). Mice were fixed for *H2*$^k$ and used at 6–18 weeks of age unless otherwise specified. All mice were housed in specific pathogen-free conditions at the Australian Phenomics

Facility and Australian Cancer Research Facility (JCSMR), with all animal procedures approved by the Australian National University Animal Ethics and Experimentation Committee.

### Flow Cytometry

Lymphoid organ suspensions were labeled with FITC-conjugated anti-CD4, anti-CD44, anti-CD45.2 (Ly5b), anti-CD69, anti-TCRβ, anti-CD3ε; PerCP-conjugated anti-CD8α; biotinylated anti-Vβ5 and anti-Vβ11; APC-conjugated streptavidin (BD PharMingen); PE-conjugated anti-CD25 and anti-CD5 (Caltag); and APC-conjugated anti-CD45.1 (Ly5a) (eBioscience). Intracellular calcium after stimulation with anti-CD3ε (500A2) was measured with a mixture of Ly5-marked splenocytes labeled with Indo-1 (Molecular Probes). anti-Foxp3-FITC intracellular staining was performed according to the manufacturer's recommended protocol (eBioscience).

### 293T Kinase Assay

The *Zap70*$^{mrd}$ mutation (ZAP-70$^{I368F}$) was introduced into a human ZAP-70-pcDNA3 construct with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All other ZAP-70 constructs as well as cDNAs coding for Lck and NTAL have been described previously (Brdicka et al., 2005). NTAL and the endogenous ZAP-70 substrate LAT have subsequently been found to be fully interchangeable in this assay. 293T cells were transfected with Lipofectamine and PLUS Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After 20 hr, cells were harvested, washed in PBS, and lysed in 60 μl 2× concentrated SDS-PAGE sample buffer. Lysates were ultracentrifuged for 30 min at 300,000 × g at 20°C and supernatants subjected to SDS-PAGE and immunoblot with antibodies against phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA), NTAL (NAP-4), ZAP-70 (2F3.2), or Lck (1F6, from J. Bolen, Millennium Pharmaceuticals, Cambridge, MA).

### Immunizations and ELISA

9- to 12-week-old mice were immunized by intraperitoneal injection with alum-precipitated ABA-CGG (Biosearch Technology) and 5 × $10^8$ whole killed *Bordetella pertussis* (Lee Labs, Becton Dickinson). Five and a half weeks later, the same mice were immunized with a combination of ABA-CGG and NP2-Ficoll (Biosearch Technology). Mice were bled 14 days after primary and 6 days after secondary immunization. For antigen-specific ELISA, serially diluted sera were adsorbed to plate-coated NP2-BSA, ABA-BSA, CGG, or sonicated *B. pertussis* (Southern Biotechnology). For total antibody ELISA, dilute serum was adsorbed to plate-bound anti-IgE (BD PharMingen) or goat anti-mouse κ light chain (Southern Biotechnology). Bound immunoglobulins were detected by alkaline phosphatase-conjugated antibodies specific to individual mouse isotypes (BD PharMingen) and quantitated by phosphatase substrate (Kirkegaard and Perry Laboratories). Sample concentrations were extrapolated from positive control standard curves.

### Autoantibodies

Serum-diluted 1/100 was applied to 12-well slides bearing permeabilized human epithelial cell HEp-2 cells (INOVA Diagnostics), washed, and then incubated with FITC-conjugated goat anti-mouse IgG (Caltag). Mounted slides were scored in blinded fashion on a Zeiss Axiophot microscope (Carl Zeiss) at 40× objective under ultraviolet light.

### Fetal Liver Chimeras

E16 embryonic liver suspensions were prepared in RPMI supplemented with 10% fetal calf serum and mixed in the appropriate ratios. 2 × $10^5$ viable cells were injected intravenously into irradiated *Rag1*$^{-/-}$ recipients, which were sacrificed for analysis 6–7 weeks later.

### Statistical Analysis

Where appropriate, one-way analysis of variance tests were performed across all groups, with Bonferroni's post-test used to determine significance as compared to the wild-type group. When data were not in an approximately normal distribution, the nonparametric Kruskal-Wallis test was used in combination with Dunn's post-test. Where p values were less than 0.05, an asterisk (*) was used to denote statistical significance. Data from two or more independent experiments were pooled in all figures. Unless otherwise indicated, bars represent the mean value of each experimental group, with circles representing individual mice.

### Supplemental Data

Three figures are available at http://www.immunity.com/cgi/content/full/27/6/912/DC1/.

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