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A novel Zap70 mutation with reduced protein stability demonstrates the rate-limiting threshold for Zap70 in T-cell receptor signalling

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Summary

Loss of ζ-associated protein 70 (Zap70) results in severe immunodeficiency in humans and mice because of the critical role of Zap70 in T-cell receptor (TCR) signalling. Here we describe a novel mouse strain generated by N-ethyl-N-nitrosourea mutagenesis, with the *reduced protein stability* (*rps*) mutation in Zap70. The A243V *rps* mutation resulted in decreased Zap70 protein and a reduced duration of TCR-induced calcium responses, equivalent to that induced by a 50% decrease in catalytically active Zap70. The reduction of signalling through Zap70 was insufficient to substantially perturb thymic differentiation of conventional CD4 and CD8 T cells, although Foxp3^{+} regulatory T cells demonstrated altered thymic production and peripheral homeostasis. Despite the mild phenotype, the Zap70[A243V] variant lies just above the functional threshold for TCR signalling competence, as T cells relying on only a single copy of the *Zap70[rps]* allele for TCR signalling demonstrated no intracellular calcium response to TCR stimulation. This addition to the Zap70 allelic series indicates that a rate-limiting threshold for Zap70 protein levels exists at which signalling capacity switches from nearly intact to effectively null.

Keywords: Foxp3; N-ethyl-N-nitrosourea mutagenesis; protein stabilization; T cell; T-cell receptor; Zap70.

Introduction

From the recent explosion of human exome sequencing experiments it is becoming increasingly apparent that every individual inherits a high mutational burden, with hundreds of rare mutations that may affect protein sequences and have a major effect on the incidence of human immune disorders.[1] Within the rare mutation pool, far more common than 'knockout' mutations are point mutations that retain at least some protein function, and yet the effect of hypomorphs is poorly understood because of a primary focus on knockout mouse models. Additional complexity to the genetic context is created by combinatorial variation, because outside consanguineous families the probability of rare mutation compound heterozygosity is greater than rare mutation homozygosity. The potential importance of rare mutations to human health makes it essential to extend the phenotype analysis of mouse models to both hypomorphs and heterozygous interactions of multiple hypomorphic alleles.[1,2]

A major protein at the crossroads between immunity and tolerance is the tyrosine kinase ζ-associated protein 70 (Zap70), which plays an essential role in activating downstream signal transduction upon T-cell receptor (TCR) engagement. These Zap70-induced signalling pathways are central to T-cell differentiation, activation and proliferation, with a loss of Zap70 in mice resulting in the absence of CD4^{+} and CD8^{+} T cells and hence severe combined immunodeficiency (SCID).[3–5] ZAP70-deficient patients present with a similar absence of CD8^{+} T cells, but CD4^{+} T cells are present in normal numbers, albeit of non-functional nature.[6] The difference between the mouse and human Zap70/ZAP70-null phenotypes lies in

Abbreviations: APC, allophycocyanin; ENU, N-ethyl-N-nitrosourea; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; Rps, reduced protein stability; SP, single positive; TCR, T-cell receptor; Treg, regulatory T

the ability of SYK to partially compensate for ZAP70-deficiency during thymic differentiation of human CD4^{+} T cells;[7,8] however, SYK cannot compensate for ZAP70 deficiency in peripheral T cells, leading to the same functional outcome of SCID in mice and humans.

Studies of a series of hypomorphic *Zap70* mutant mice with graded defects in TCR signalling significantly extended the understanding of the fine-tuned balance between tolerogenic and immunogenic signalling in T cells by revealing phenotypes that would not be predicted based on the simple SCID phenotype of knockout mice. Indeed, in various hypomorphic mutant strains, immune deficiency was associated with immune hyper-activation and/or autoimmunity, ranging from hyper-IgE,[9,10] anti-DNA[9] and rheumatoid factor autoantibody production,[11,12] to overt development of autoimmune arthritis.[12] The presentation of immune dysregulation in these mutant mouse strains was associated with intermediate TCR signalling, showing that the degree of immune deficiency determines the paradoxical development of autoimmunity, i.e. by impairing negative selection and/or by disturbing regulatory T (Treg) cell function, disturbing the homeostatic equilibrium between immunogenic and tolerogenic T-cell functions.[9,13,14]

In the present study, we describe a novel hypomorphic *Zap70* mutant mouse strain generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, Zap70[rps] mice. The *reduced protein stability* mutation results in an A243V change, which causes a ~ 55% reduction in Zap70 protein levels. Here we demonstrate that a 55% reduction in functional Zap70 protein results in only mild TCR signalling and cellular phenotypes, whereas a further 50% reduction in Zap70 protein levels generated by compound heterozygosity of hypomorphic alleles demonstrates near-normal positive selection but no peripheral intracellular calcium responses to TCR stimulation. These data demonstrate that combinatorial association of multiple hypomorphic alleles can generate unexpected phenotypes once the net genetic burden rises above set thresholds.

Materials and methods

Mice

Foxp3[GFP] mice[15] were backcrossed to the C57BL/6 background. *Zap70*[mrt] mice are described in the accompanying paper[16]. To generate the *Zap70*[rps] strain, founder C57BL/6 male mice were treated with 100 mg/kg ENU and bred to *Foxp3*[GFP] females. First-generation male offspring were bred back to *Foxp3*[GFP] females to produce the second-generation offspring, which were in turn inter-crossed to produce the third generation for phenotype screening. Phenotypic screening involved flow cytometric analysis for CD4 (anti-CD4 antibody) and Foxp3

(GFP), and identification of individuals with values for the percentage of GFP^{+} cells within the CD4^{+} population that were more than two standard deviations from the norm. The identified variant offspring were inter-crossed to produce the C57BL/6.*Foxp3*[GFP].*Zap70*[rps] mutant mouse strain. Experimental mice were age-matched and housed under specific pathogen-free conditions. All mice were used after ethics approval of the University of Leuven mouse facility.

Molecular biology

The *rps* mutation identified by all-exome sequencing was confirmed by Sanger sequencing. Using primers that flank the *rps* mutation (forward primer 5′-GAC AAG GCT GGC AAG TAC TGC ATC-3′ and reverse primer 5′-TGT CAC GTC TCA ACG CTG AGG TG-3′) a 400-bp fragment was amplified and cloned into the pCR[®]4-TOPO[®] vector (Invitrogen, Carlsbad, CA) for sequencing.

Zap70[rps] mice were genotyped using the Amplifluor SNP genotyping assay based on PCR amplification with tailed allele-specific primers, a common reverse primer and fluorescence-labelled (JOE-FAM) Amplifluor universal primers (Millipore, Billerica, MA). Primers were designed with the ASSAYARCHITECT software (Millipore) and were 5′-GA AGG TCG GAG TCA ACG GAT TTG GAG TAC CTG AAG CTG AAG GC-3′ (wild-type), 5′-GAA GGT GAC CAA GTT CAT GCT GTG GAG TAC CTG AAG CTG AAG GT-3′ (*rps*) and the common reverse primer was 5′-CTG TTG GGA CAG ACC TCC TT-3′. Fluorescence end-point measurement was performed using the Taqman RT-PCR 7300 (Applied Biosystems, Foster City, CA).

CD4^{+} CD8^{−} and CD4^{−} CD8^{+} splenocytes were sorted after enrichment with an EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies, Vancouver, BC, Canada). RNA was purified using an RNeasy micro kit (Qiagen, Hilden, Germany) and was reverse transcribed with random hexamers (Life Technologies, Carlsbad, CA) and M-MLV (Invitrogen). Quantitative PCR was performed with the StepOnePlus (Life Technologies) and TaqMan Fast Universal PCR Master Mix (Life Technologies) for *Zap70* (Mm.PT.56a.12463755, IDT DNA) and *β-actin* (Mm.PT.56a.33540333, IDT DNA). Relative gene expression was determined by the $2^{-\Delta\Delta C_t}$ method[17] and normalized to the average of wild-type CD4^{+} T cells.

Flow cytometry

Cell surface staining and flow cytometric analysis were performed using the following antibodies: anti-B220-phycoerythrin (PE) -Cy5 (1/400) (RA3-6B2) or PE-Cy7 (1/400) (RA3-6B2), and anti-CD3e-allophycocyanin (APC) (1/400) (145-2c11), anti-CD4-APC (1/400) (GK1.5) or APC-H7 (1/400) (GK1.5) or Peridinin chlorophyll protein

(PerCP)-Cy5.5 (1/400) (GK1.5) or eFluor450 (1/400) (GK1.5), anti-CD8-APC-eFluor780 (1/400) (53-6.7) or PerCP-Cy5.5 (1/400) (53-6.7), anti-CD25-APC (1/250) (PC61.5) or PE (1/250) (PC61.5), anti-CD62L-PE-Cy7 (1/250) (MEL-14) and anti-CD69-PE-Cy5.5 (1/250) (H1.2F3) or PE-Cy7 (1/250) (H1.2F3) (all eBioscience, San Diego, CA, unless indicated otherwise). Intracellular staining with anti-Foxp3-APC (1/100) (eBioscience), anti-Ki67-PE (1/50) (BD) and anti-Zap70-PE (1/400) (1E7.2; eBioscience) antibodies was performed following fixation and permeabilization using the reagents from the eBioscience Foxp3 staining kit. Data were collected by BD Canto I and Canto II. Cell sorting was performed on splenocytes stained for anti-CD4-PE-Cy7 (GK1.5, eBioscience) and anti-CD8α-APC-eFluor780 (eBioscience) on a BD FACS Aria III.

T-cell stimulation

Splenocytes were resuspended in 10% complete RPMI-1640 media at 1×10^7 cells/ml in round-bottomed 96-well plates (Greiner Bio-one, Frickenhausen, Germany). Cells were stimulated with plate-bound anti-CD3 (eBioscience) and anti-CD28 (eBioscience) antibodies at 37° for 24 hr before harvesting for flow cytometry as described above.

Calcium flux assays were performed on freshly isolated splenocytes. Before analysis cells were loaded with Fluo-4 (0·8 μg/ml; Invitrogen) and Indo-1 (2 μg/ml; Invitrogen), and stained with anti-B220-APC (1/400) (RA3-6B2), anti-CD3-biotin (145-2c11), anti-CD11b-PE (1/400) (M1/70), anti-Ly-6G-APC (1/400) (Gr-1), anti-MHC class II-PE (1/400), anti-NK1.1-APC (1/400) (PK136), anti-TER-199-PE (1/400) (TER-119), and either anti-CD4-biotin (1/200) (RM4-5) and anti-CD8-APC-Alexa780 (1/400) (53–6·7) or anti-CD4-APC-Cy7 (1/400) (GK1.5) and anti-CD8-biotin (1/200) (53–6·7) (all eBioscience unless indicated otherwise). A base line level of Fluo-4 and Indo-1 fluorescence was acquired at 37° on a BD Aria III before the addition of streptavidin (Sigma, St Louis, MO) to cross-link anti-CD3 and anti-CD4. Immediately following streptavidin addition, acquisition of flow data was reinitiated for an additional 500 seconds.

Statistics and modelling

Statistical analysis was performed using Prism (Graphpad, San Diego, CA). A significance threshold of 5% in a Mann–Whitney U-test or a Kruskal–Wallis test (with Dunn's correction for multiple comparisons) was maintained throughout the study. P-values were obtained by comparison of the Zap70 mutant with wild-type control values (and between $Zap70^{rps}$ and $Zap70^{mrt/wt}$ in Fig. 5). Homology alignment was performed using Clustal Omega, BLAST and Jalview.[18] Pymol Molecular Graphics System (Version 1.5.0.4 Schrödinger, LLC, Portland, OR) was used for the modelling of the Zap70 structure.

Results

Identification of the reduced protein stability mutation in Zap70 and the resulting alteration in regulatory T-cell differentiation

The ENU-exposed C57BL/6 mice were bred to $Foxp3^{GFP}$ females to generate a standard F_2 intercross pedigree[19] allowing screening of ENU mutants with altered numbers of Foxp3+ Treg cells in the blood. Within one pedigree, individuals were identified with an increased number of peripheral blood GFP+ Treg cells. Inter-crossing of affected individuals resulted in the reduced protein stability (rps) mutant strain, which consistently demonstrated increased numbers of Treg cells in the blood by 5–6 weeks of age (Fig. 1a). All-exon sequencing of affected individuals identified a C→T nucleotide substitution at nucleotide 36778405, which was confirmed by Sanger sequencing (Fig. 1b). This mutation resulted in an alanine to valine change at amino acid 243 in a region of the Zap70 protein sequence highly conserved across mammals (Fig. 1c) and partially conserved across related kinases in mice (Fig. 1d). The A243V change is located within the C-terminal SH2 domain, one of the two domains involved in the docking of Zap70 to phosphorylated immunoreceptor tyrosine-based activation motif on the TCR-ζ peptide (Fig. 1e–g).

Due to the sensitivity of T-cell differentiation to defects in TCR signalling, we next investigated the impact of the $Zap70^{A243V}$ variant on thymic differentiation. Unlike mice bearing a null allele of $Zap70$,[5] $Zap70^{rps}$ mice did not have a major defect in positive selection. The overall thymic cellularity was unchanged in $Zap70^{rps}$ mice (data not shown) and the percentage of CD4+ CD8+ double-positive thymocytes remained intact, while the frequency of post-selection CD69+ double-positive thymocytes was reduced by ~ 20% (Fig. 2a–c). Similarly, a small impairment in lineage selection was observed, with a ~ 25% decline in the number of CD4+ single-positive thymocytes (Fig. 2d and Supplementary material, Fig. S1a), whereas CD8+ single-positive thymocytes were unchanged (Fig. 2e and Supplementary material, Fig. S1b). In addition, positive selection into the Foxp3+ regulatory T-cell lineage was reduced by ~ 30% in $Zap70^{rps}$ mice (Fig. 2f and Supplementary material, Fig. S1c). Non-complementation of the $Zap70^{rps}$ mutation was observed upon inter-crossing with the $Zap70^{mrt}$ strain (which is a severe hypomorph), as $Zap70^{rps/mrt}$ heterozygote mice bore defects in thymocyte differentiation ranging between that of the parental strains (Fig. 2b–f and Supplementary material, Fig. S1a–c). The mild defect in positive selection observed in the thymus was perpetuated in the periphery, with a ~ 25% drop in both CD4+ and CD8+ T cells in the spleen of $Zap70^{rps}$ mice relative to wild-type mice (Fig. 3a–c and Supplementary material, Fig. S1d, e) but similar total
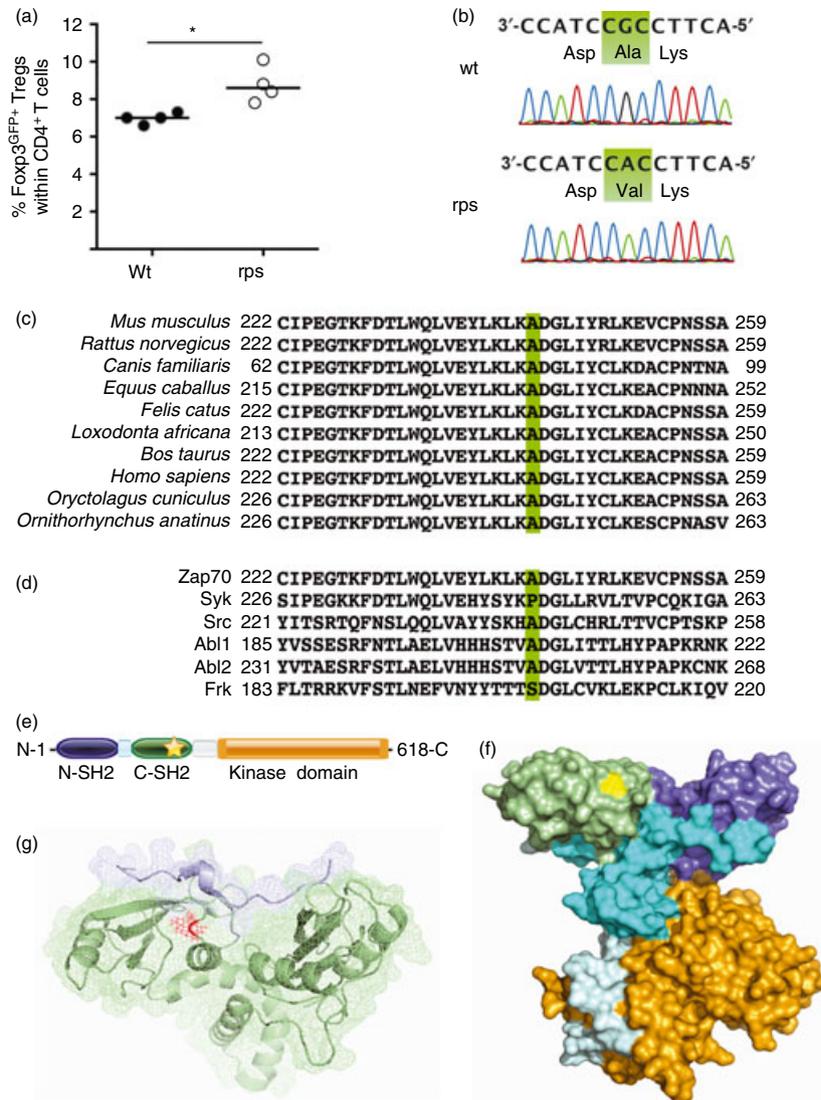
Figure 1. Increased peripheral regulatory T (Treg) cell frequencies in Zap70[rps] mice with an A243V in the C-terminal SH2-domain of Zap70. (a) Peripheral blood lymphocytes from wild-type and rps mutant mice were assessed for the percentage of Foxp3[GFP+] Treg cells within the CD4^{+} T-cell compartment at 5–6 weeks of age. (b) Sanger sequencing of Zap70 in wild-type and rps mutant mice confirmed a C to T mutation at location 36778405, resulting in an alanine to valine change at amino acid 243. (c) Conservation of the rps mutation site between the mouse, rat, dog, horse, cat, elephant, bovine, human, rabbit and platypus homologous mammal sequences. (d) Partial conservation of the *rps* mutation site between related murine kinases, Zap-70, Syk, Src, Abl1, Abl2 and Frk. (e) Location of the A243V amino acid change (star) within the domain structure of Zap70. Identified are the N-terminal SH2 domain (purple), interdomain A (blue), C-terminal SH2 domain (green), interdomain B (grey) and kinase domain (orange). (f) Location of the A243 residue (yellow) within the cloud structure of Zap70. Identified are the N-terminal SH2 domain (purple), interdomain A (blue), C-terminal SH2 domain (green), interdomain B (grey) and kinase domain (orange). (g) Modelling of SH2 domains (green) in contact with TCR-ζ peptide (blue). The A243 residue is shown in red at the binding interface. Structures from PyMOL. *$P < 0.05$.

splenocyte numbers (data not shown). Again, the *Zap70[rps]* mutation was not complemented by the Zap70[mrt] mutation (Fig. 3a–c and Supplementary material, Fig. S1d, e), reinforcing the conclusion that T-cell defects in the *rps* strain are caused by the Zap70[A243V] variant. Finally, consistent with other *Zap70* hypomorphs[9] and the robust niche-filling capacity of Treg cells[20], splenic Treg cell numbers rebounded to normal levels despite the ~ 30%

reduction in thymic numbers, an effect which, when combined with the mild CD4^{+} lymphopenia, resulted in a relative enrichment of Treg cells within CD4^{+} T cells (Fig. 3d). In total numbers, the rebound was less obvious, but it was still apparent as the initial ~ 70% decline in *Zap70[rps]* thymic Treg cell number output ended up in only a ~ 30% decrease in peripheral numbers (Supplementary material, Fig. S1c,f). This relative enrichment
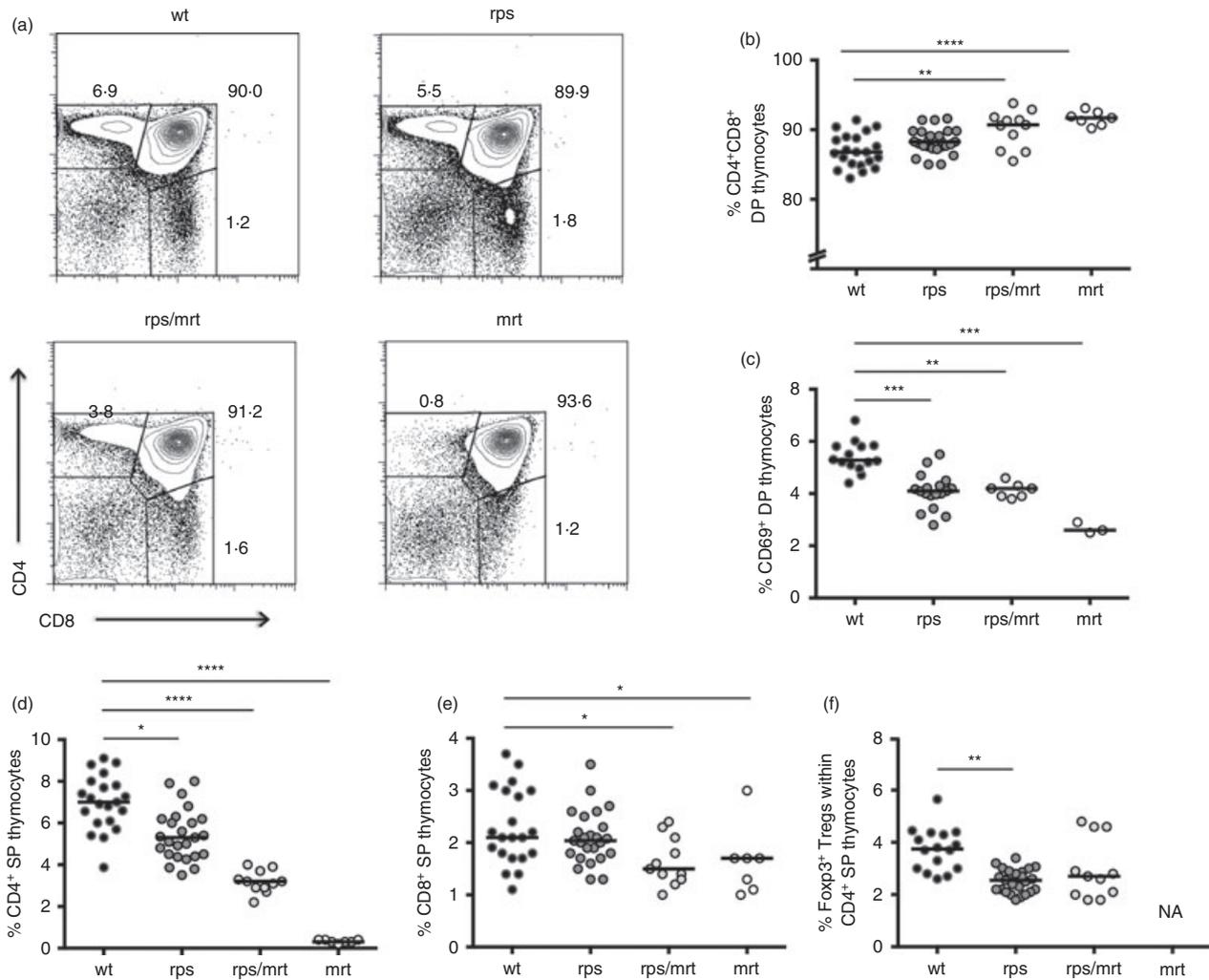
Figure 2. Reduced positive selection of regulatory T cells in mice bearing the Zap70[A243V] variant. Wild-type, Zap70[rps], Zap70[rps/mrt] and Zap70[mrt] mice were assessed for thymic function at 5–13 weeks of age for T-cell subpopulations (n = 14–21, 18–25, 7–11, 3–7 mice/group). (a) Representative flow cytometry plots for CD4 versus CD8. (b) Percentage of CD4$^+$ CD8$^+$ double-positive (DP) thymocytes in mice of each genotype (median with individual values shown). (c) Percentage of DP thymocytes expressing CD69 in mice of each genotype (median with individual values shown). (d) Percentage of CD4$^+$ single-positive (SP) thymocytes in mice of each genotype (median with individual values shown). (e) Percentage of CD8$^+$ SP thymocytes in mice of each genotype (median with individual values shown). (f) Percentage of Foxp3$^+$ regulatory T cells within the CD4$^+$ SP population in mice of each genotype, with the exception of Zap70[mrt] mice where the parent population was absent (NA) (median with individual values shown). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

was probably responsible for the phenotype of increased number of peripheral blood GFP$^+$ Treg cells on which the *rps* strain was first identified and which remains constant with age (Supplementary material, Fig. S2).

The Zap70[A243V] variant demonstrates reduced protein stability and impaired calcium signalling

To determine the mechanism of activity of the *Zap70[rps]* mutation the relative expression of the Zap70[A243V] variant was tested at the mRNA and protein levels. Complementary DNA was generated from sorted peripheral CD4$^+$ and CD8$^+$ T cells and the relative expression of Zap70

was assessed by quantitative PCR. No differences were observed between wild-type and *Zap70[rps]* T cells, either CD4$^+$ or CD8$^+$, indicating that the *rps* mutation does not affect mRNA expression or longevity (Fig. 4a). To determine the effect of the Zap70[A243V] variant on protein expression, Zap70 expression was measured by flow cytometry in CD4$^+$ and CD8$^+$ T cells of both the thymus and spleen. In the thymus, CD4$^+$ and CD8$^+$ single-positive T cells displayed a 50–70% reduction in Zap70 protein levels in *Zap70[rps]* mice (Fig. 4b,c). By contrast, Zap70 expression was almost undetectable in the same population from *Zap70[mrt]* mice, in which a major disruption to the catalytic site results in rapidly degraded
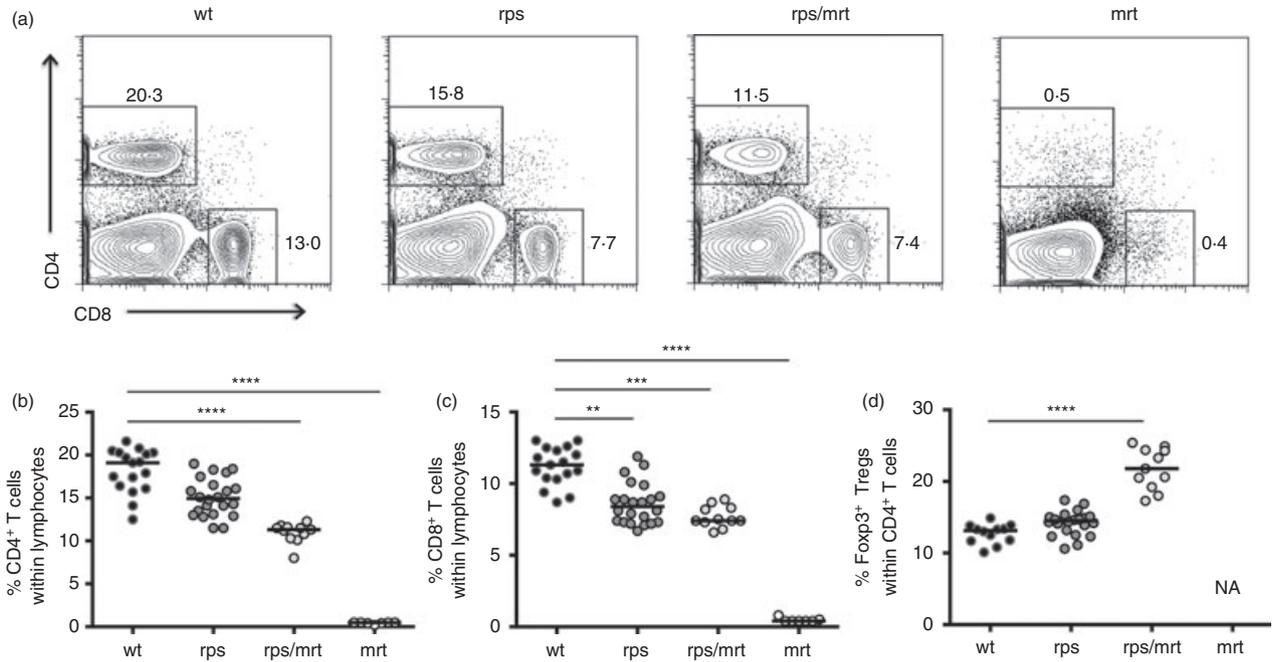
Figure 3. Mild lymphopenia and enrichment of regulatory T cells in Zap70^rps mice. Wild-type, Zap70^rps, Zap70^rps/mrt and Zap70^mrt mice were assessed at 5–13 weeks of age for T-cell subpopulations in the spleen ($n = 12$–17, 22, 11, 7 mice/group). (a) Representative flow cytometry plots for CD4 versus CD8. (b) Percentage of CD4+ T cells within the lymphocyte gate in mice of each genotype (median with individual values shown). (c) Percentage of CD8+ T cells within the lymphocyte gate in mice of each genotype (median with individual values shown). (d) Percentage of Foxp3+ Treg cells within the CD4+ T-cell population in mice of each genotype, with the exception of Zap70^mrt mice where the parent population was absent (NA) (median with individual values shown). **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

protein[16]. Compound heterozygotes between the *Zap70^rps* and *Zap70^mrt* strains showed a further ~ 50% reduction in Zap70 protein levels, to ~ 30% of wild-type levels (Fig. 4c). A reduction of ~ 55% in Zap70 protein level was also observed in the spleen of *Zap70^rps* mice, indicating that peripheral compensation mechanisms were not effective (Fig. 4d,e). Together, these results demonstrate that the *rps* mutation results in a Zap70 variant with reduced protein stability, titrating down the available Zap70 protein levels to ~ 45% across organs and lineages.

To determine the impact of reduced Zap70 levels on downstream signalling, CD4+ and CD8+ T cells from wild-type and *Zap70^rps* mice were stimulated *in vitro* for 24 hr. For both *Zap70^rps* CD4+ and CD8+ T cells, the up-regulation of early activation markers (CD25+ CD69+ T cells) was slightly reduced from that of wild-type cells (Fig. 5a), similar to that observed in *Zap70^mrt/wt* mice, in which functional Zap70 is reduced by ~ 45%. Notably,

reliance on a single copy of *Zap70^rps* in combination with the severely hypomorphic *Zap70^mrt* allele (in *Zap70^rps/mrt* T cells) resulted in a more significant drop in T-cell activation, although still less than that observed in *Zap70^mrt* mice (Fig. 5a). To determine the quantity and quality of the immediate TCR signalling response, wild-type and *Zap70^rps* T cells were challenged in a Ca^{2+} flux assay. Following loading with Ca^{2+}-sensitive dye and recording of baseline fluorescence, wild-type and *Zap70^rps* CD4+ T cells were stimulated via cross-linking of CD3 and CD4, respectively. No differences in initial activation status between *Zap70^rps*, *Zap70^wt/mrt* and wild-type CD4+ T cells were observed, whereas *Zap70^rps/mrt* CD4+ T cells showed significantly increased proportions of activated cells (Supplementary material, Fig. S3a). Individual Ca^{2+} signalling results showed a uniform response, excluding the possibility that the observed difference was due to population heterogeneity (Supplementary material, Fig. S3b). Relative

Figure 4. Decreased Zap70 protein levels in Zap70^rps mice. (a) CD4+ and CD8+ T cells were purified from the spleen of wild-type and Zap70^rps mice. Relative Zap70 expression was assayed through quantitative PCR measurement, relative to *β-actin* ($n = 3$/group). (b–e) The thymus and spleen of wild-type, Zap70^rps, Zap70^rps/mrt and Zap70^mrt mice was stained with antibodies against CD4, CD8 and Zap70 for flow cytometric analysis ($n = 7$–14, 7–16, 4, 4–11, 4–7/group). (b) Representative flow cytometry plots and (c) mean fluorescence intensity (MFI) for CD4+ and CD8+ single-positive thymocytes (median with individual values shown). (d) Representative flow cytometry plots and (e) mean fluorescence intensity for CD4+ and CD8+ splenocytes (median with individual values shown). *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.
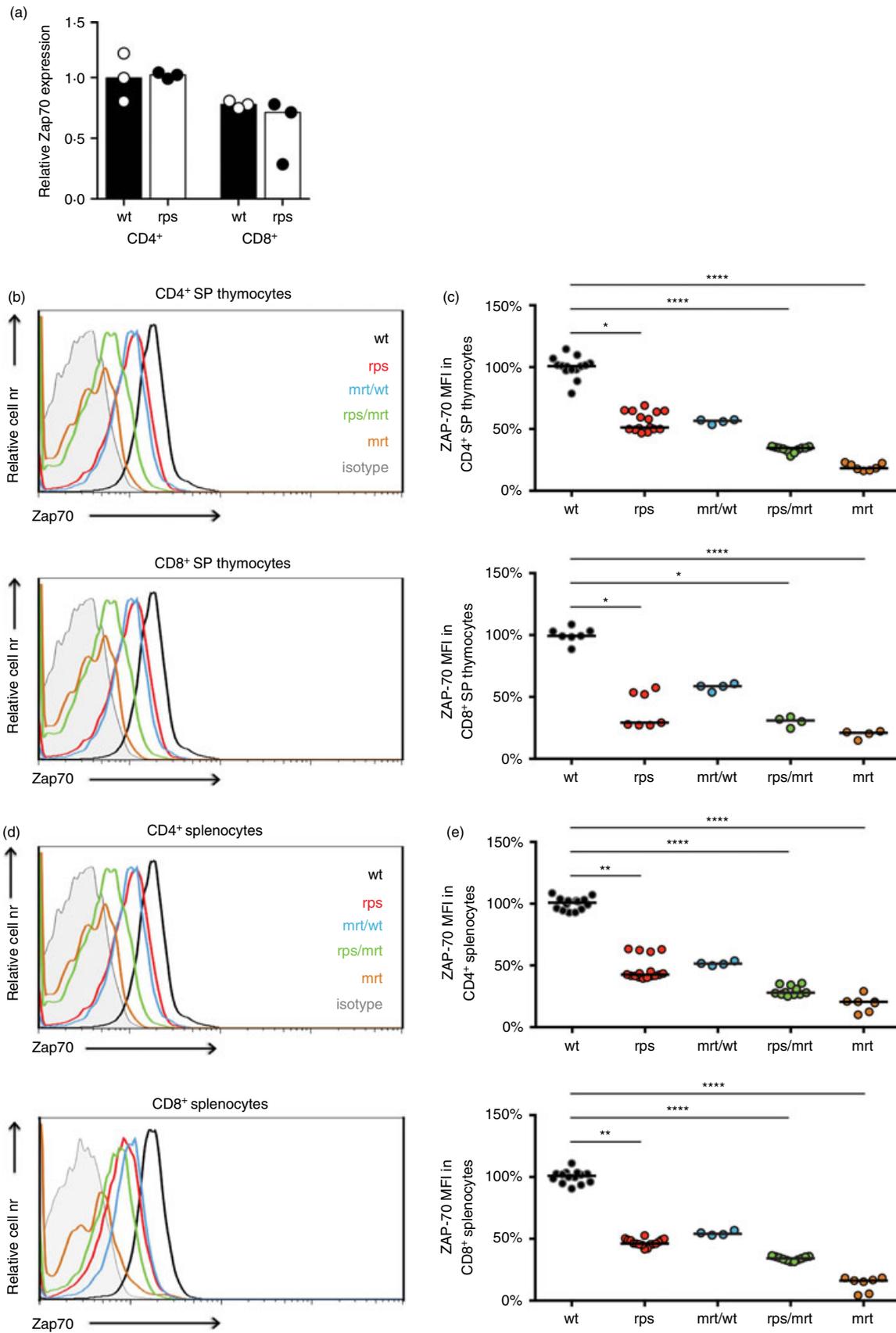
to wild-type CD4$^+$ T cells, *Zap70^{rps}* CD4$^+$ T cells showed a mild impairment of TCR signalling (Fig. 5b), with no significant reduction in peak intracellular Ca^{2+} (Fig. 5c), but a reduced duration of elevated intracellular Ca^{2+} (Fig. 5d). Hence, despite a reduction to ∼ 55% of wild-type Zap70 protein levels, TCR signalling remained relatively unchanged in *Zap70^{rps}* T cells. These results were very similar to *Zap70$^{wt/mrt}$* T cells, with ∼ 45% of functional Zap70 levels. Strikingly, however, further reduction of functional Zap70 protein levels to ∼ 30% of wild-type levels (observed in *Zap70$^{rps/mrt}$* T cells) led to a complete blockade of effective Ca^{2+} flux in response to TCR stimulation (Fig. 5b–d). This discrepancy between *Zap70^{rps}* T cells and *Zap70$^{rps/mrt}$* T cells demonstrates a non-linear relationship between Zap70 protein availability and TCR signalling events, as exemplified by the sigmoidal curve of Zap70 protein levels versus Ca^{2+} flux peaks (Fig. 5e) or versus general T-cell activation (Fig. 5f). Analysis of this relationship suggests a relatively narrow protein concentration range in which near-normal TCR signalling switches into an effectively non-functional state.

Discussion

In the present study, we characterized a novel *Zap70* hypomorphic mutant mouse strain, *Zap70^{rps}* mice, in which an A243V change resulted in a 55% reduction in the Zap70 protein concentration. The phenotypic changes induced by ∼ 50% reduction in Zap70 protein, either in *Zap70^{rps}* mice or mice heterozygous for the *mrt* mutation, proved relatively subtle, with a few notable exceptions. In both genetic contexts, TCR signalling remained largely intact, with normal peak intracellular Ca^{2+} achieved following TCR stimulation but a reduction in the duration of elevated intracellular Ca^{2+}. This defect can be explained by the ∼ 50% reduction in Zap70 protein levels in *Zap70^{rps}* T cells, as *Zap70$^{mrt/wt}$* T cells, which have one normal fully-function copy of Zap70 and one severely hypomorphic and rapidly degraded copy of Zap70, have an indistinguishable phenotype. Nevertheless, it is conceivable that the A243V change also results in impaired function of the remaining protein. The two SH2 domains of Zap70 are involved in the binding of Zap70 to the TCR-ζ chain, following the phosphorylation of the immunoreceptor tyrosine-based activation motif by Lck. This binding releases Zap70 from its auto-inhibited conformation, allowing subsequent signal transduction.[4] Crystal structure studies indicate that Y238 and K242 are closely associated with the phosphotyrosine of the immunoreceptor tyrosine-based activation motif peptide,[21] suggesting that changes to the neighbouring 243 residue could impair docking of Zap70 to CD3ζ. Such a function would of necessity be minor, because otherwise the additive effects of reduced protein level and impaired protein function would be predicted to be greater than that of a similar reduction in protein level alone.

Regardless of the molecular mechanism by which Zap70 signalling is affected, the mild alteration in signalling capacity of *Zap70^{rps}* and *Zap70$^{mrt/wt}$* T cells produced only small decreases in the TCR-dependent processes of positive selection and peripheral T-cell activation. The TCR-dependent process, which demonstrated the most substantial defect due to the 50% reduction in Zap70 protein levels, was the positive selection of Foxp3$^+$ Treg cells. There are several reasonable hypotheses for this. First, thymic Foxp3 induction in Treg cell precursors is a positive selection event requiring an elevated level of TCR signalling[22] and qualitatively different downstream mediators of signal transduction,[23] such that reduction of the kinase function may differentially impact Treg cells. Alternatively, Treg cells have been demonstrated to be dependent on a non-kinase function of Zap70 for activation of Rap1.[24] As Rap1 is involved in thymic Treg cell induction,[25,26] it is plausible that partial loss of Zap70 protein has a stronger effect on Treg cell induction via reduction of both the kinase-dependent and Rap1-dependent Zap70 signalling pathways.

One interpretation of the resilience of TCR-dependent signalling to substantial losses in Zap70 protein could be that the Zap70 signalling pathway requires very little functional protein to signal. In this scenario it could be predicted that further reduction in Zap70 protein levels would lead to a progressive decline in total Zap70-dependent signalling. In contrast to this supposition, however, *Zap70$^{rps/mrt}$* T cells demonstrated a profound defect in TCR signalling. In *Zap70$^{rps/mrt}$* T cells the functional level of Zap70 protein was reduced to ∼ 30% of normal levels, as a result of the compound effect of one allele of *reduced protein stability* producing half the normal Zap70 quotient and one allele (*mrt*) largely unable to produce functional levels of Zap70. The phenotype of these *Zap70$^{rps/mrt}$* T cells was striking. Positive selection was essentially intact, however peripheral T-cell signalling was essentially absent. No intracellular Ca^{2+} flux was observed upon TCR-stimulation and even 24 hr of stimulation only resulted in very minor up-regulation of CD69 and CD25, consistent with just a 'trickle' of signalling capacity being left intact. It is tempting to speculate that the largely intact nature of positive selection in *Zap70$^{rps/mrt}$* mice, in contrast to the defective peripheral signalling, is due to cooperation between remnant Zap70 with Syk. In human thymocytes, SYK is expressed at a higher level than in mouse thymocytes, and can substitute for ZAP70 until SYK expression is lost in the periphery.[7] It is plausible that murine Syk, expressed at lower levels than SYK, cannot substitute for complete Zap70-deficiency but can substitute for the ∼ 70% Zap70-deficiency observed in *Zap70$^{rps/mrt}$* mice,[27] until Syk is lost in the periphery. Alternatively, this feature may simply emerge as a result of the lower signalling
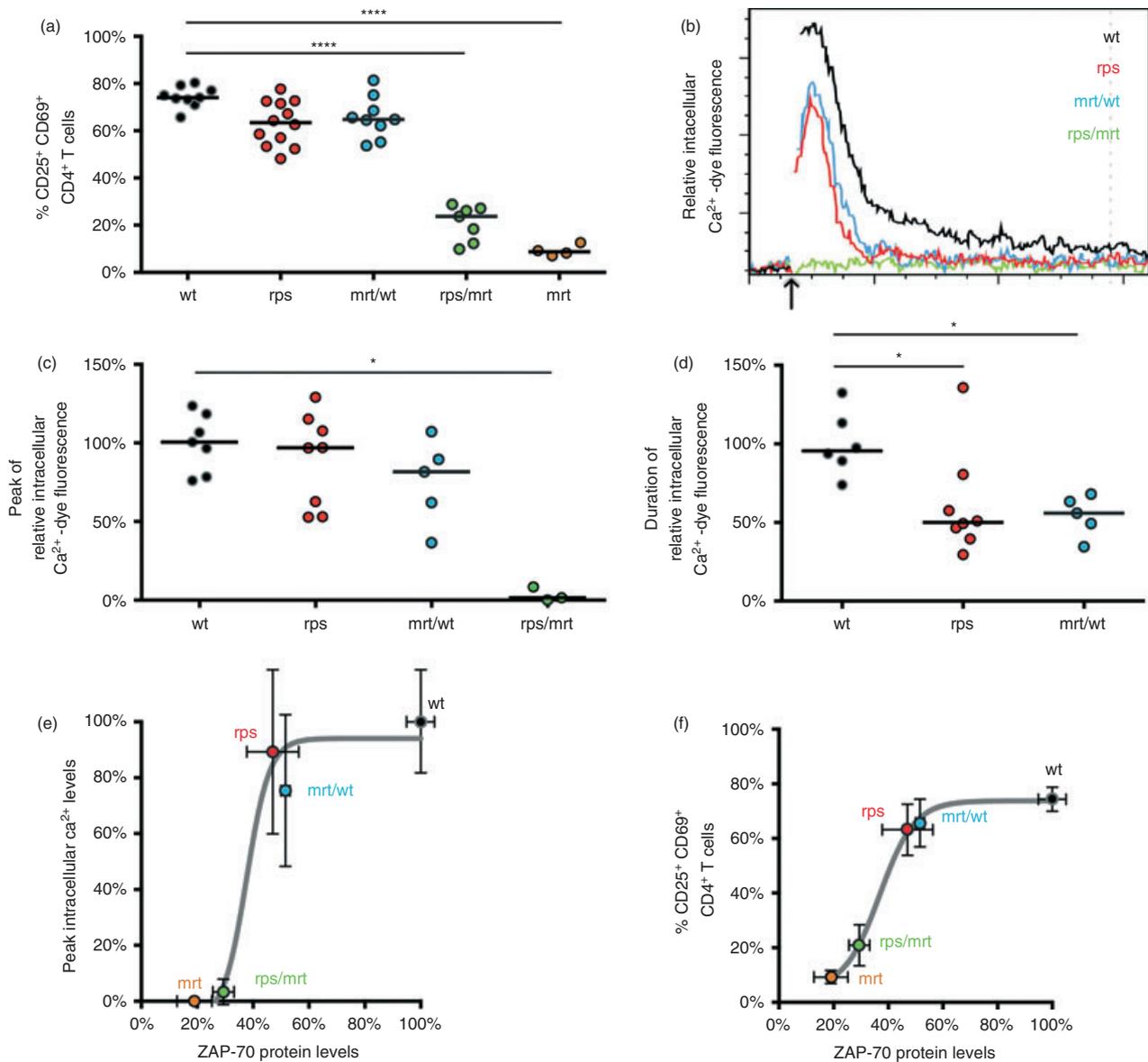
Figure 5. Reduced Zap70 protein levels in Zap70^{rps} CD4$^+$ and CD8$^+$ T cells leave T-cell receptor (TCR) signalling above the critical threshold for activity. (a) Splenocytes from wild-type, Zap70^{rps}, Zap70$^{mrt/wt}$, Zap70$^{rps/mrt}$ and Zap70^{mrt} mice were stimulated for 24 hr with anti-CD3 and anti-CD28 before measurement of activation via CD25 and CD69 up-regulation in CD4$^+$ T cells (n = 9, 12, 9, 7, 4 mice/group, median and individual values shown). (b) CD4$^+$ splenocytes from wild-type, Zap70^{rps}, Zap70$^{mrt/wt}$, and Zap70$^{rps/mrt}$ mice were loaded with Ca^{2+}-dependent dye and assessed by flow cytometry for intracellular Ca^{2+}. The arrow indicates the addition of streptavidin to cause the cross-linking of CD3 and CD4. Representative histograms are shown gated on CD4$^+$ T cells. n = 7, 8, 5, 3 mice/group (c) Relative peak intracellular Ca^{2+}-dependent dye fluorescence (median and individual values shown) and (d) duration of elevated intracellular Ca^{2+}-dependent dye fluorescence in the cells described in (b) (median and individual values shown). (e, f) Relationship between Zap70 protein levels (relative to wild-type T cells based on data in Fig. 4e) and (e) peak intracellular Ca^{2+} levels following TCR stimulation (relative to wild-type T cells based on data in part c of this figure) or (f) activation (as depicted in panel a of this figure) in wild-type, Zap70^{rps}, Zap70$^{mrt/wt}$, Zap70$^{rps/mrt}$ and Zap70^{mrt} mice for CD4$^+$ splenocytes. Mean and standard deviation for each genotype is shown, with the Boltzmann sigmoidal function of best fit. *$P < 0.05$; ****$P < 0.0001$.

requirement of positive selection versus peripheral T-cell activation.[9,22] Regardless, it is striking that the reduction of Zap70 protein from 100% to 50% has almost no impact on TCR signalling, whereas further reduction from 50% to 30% results in an essentially complete blockade. This sigmoidal relationship between protein concentration and protein function constitutes a switch-like function, analogous to the dose–response function of TCR ligand affinity, which oscillates from 'all on' to 'all off' over a relatively small affinity range.[28,29]

Finally, these observations serve as a case study for the emergent phenotypes that can develop in complex genetic

contexts. A recent study of publicly available exome sequencing data concluded that about 2% of the human population carries a rare, non-synonymous (protein sequence-altering) variant in any given gene, most of which are rare variants, i.e. found in < 1% of the population.[1] Assuming *ZAP70* is not exceptional, about four individuals per 10 000 will inherit compound heterozygous mutations in *ZAP70*. Our results here suggest that most of these combinations will have no observable phenotype, as the change induced by ∼ 50% reduction in functional Zap70 in mice is well within the normal variation observed in humans. At the other end of the spectrum are ZAP70 deficiencies sufficient to cause SCID.[6] Genetic defects within these extremes have been observed in humans, such as a case of hypomorphic *ZAP70*, which resulted in late onset immunodeficiency.[30] Notably, most of these mutations affect protein stability of ZAP70,[6,31–33] functioning in a similar manner to the *rps* mutation described here. Since we know from mouse studies that Zap70 mutations in the intermediate range produce an admixture of immunodeficiency and autoimmunity,[9–12] it is surprising that such cases have not been observed in humans, as genetic combinations that fall within the mid-range of the mutational spectrum should be more common than those at the extremes. To some degree this may be due to ascertainment bias in genetic diagnosis; however, the recent growth in all-exome sequencing approaches[1,2] should negate such effects. Our results here provide an alternative explanation for the absence of clinical phenotypes representative of intermediate defects: namely that the window for intermediate defects is narrow, such that most defects in ZAP70 will fall either into the phenotypically normal or phenotypically null range. The extrapolation of these results on Zap70 to other genes would likewise provide an explanation for the vast majority of individuals having normal healthy phenotypes despite a surprisingly high genome-wide inherited mutational burden.

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Disclosures

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Total numbers of thymic and splenic T-cell subpopulations.

Figure S2. Increased peripheral regulatory T (Treg) cell frequencies in Zap70[rps] mice remain constant over age.

Figure S3. Individual cellular Ca^{2+} flux responses are uniform and unaffected by activation status.