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A ZAP-70 kinase domain variant prevents thymocyte-positive selection despite signalling CD69 induction

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Summary

Quantitative reductions in T-cell receptor (TCR) signalling are associated with severe immunodeficiency, yet in certain cases can lead to autoimmunity. Mutation of the tyrosine kinase ZAP-70 can cause either of these outcomes, yet the limits of its signal transducing capacity are not well defined. To investigate these limits we have made use of *mrtless*: a chemically induced mutation of *Zap70* associated with T-cell deficiency. Unlike cells devoid of ZAP-70, *mrtless* thymocytes showed partial induction of CD5 and CD69, and were sensitive to TCR stimulation with a dose–response shifted approximately 10-fold. However, essentially no T cells were able to compensate for the *mrtless* mutation and mature beyond the $CD4^+$ $CD8^+$ stage. This outcome contrasts with a ZAP-70 Src Homology 2 domain mutant strain, where high-affinity self-reactive TCR are positively selected rather than deleted. We discuss these data with respect to current models of TCR signalling in thymocyte selection.

Keywords: genetics; T cells; thymic selection.

Introduction

Tyrosine kinases are essential for T-cell antigen recognition, and propagate signals from the T-cell receptor (TCR) by phosphorylating and activating downstream substrates. The two most critical tyrosine kinases involved in TCR signalling are ZAP-70 and Lck. Lck is recruited to the TCR by CD4 and CD8 co-receptors, activating ZAP-70 by direct phosphorylation and by creating docking sites on CD3 subunits to which the tandem Src Homology 2 (SH2) domains of ZAP-70 bind. Mice lacking ZAP-70 exhibit a complete arrest of $\alpha\beta$ T-cell development at the $CD4^+$ $CD8^+$ stage: a point at which cells are selected for TCRs with low avidity recognition of self-peptide/

Abbreviations: Lck, lymphocyte protein tyrosine kinase; Syk, spleen tyrosine kinase; SH2, Src Homology 2; HEL, hen egg lysozyme; Cbl, casitas B-lineage lymphoma; SLAP, Src-like adaptor protein; Rap1, RAS-related protein-1a

MHC complexes.[1–3] Residual development of $CD4^+$ $CD8^-$ T cells occurs in ZAP-70-deficient humans as a result of the compensatory action of the related kinase SYK,[4] although these cells are unresponsive to TCR stimulation.[4–7] Mice deficient for both ZAP-70 and Syk have an arrest of T-cell development at the double-negative 3 (DN3, $CD4^-$ $CD8^-$ $CD44^-$ $CD25^+$) stage, where thymocytes are selected for productive rearrangement of the *Tcrb* locus.[8]

Null alleles of ZAP-70 and other TCR signalling molecules prevent T-cell maturation, but much less is known about how inherited variants causing quantitative changes in TCR signalling affect T-cell function. This is significant from both a mechanistic and a clinical perspective, because subtle single amino acid substitutions represent the most common functional genetic variation in humans. In the case of human *ZAP70*, 0·338% of alleles in a cohort of 6503 exomes were found to be missense variants (http://evs.gs.washington.edu/EVS/), and more than a quarter of these are predicted to be damaging (14/44 annotated as probably damaging by PolyPhen-2). At these frequencies, more than one person in every 10 000 could be expected to inherit a combination of deleterious mutations in *ZAP70*, although which of these combinations will confer a clinical phenotype is more difficult to predict.[9]

It is clear from studies of null mutations in mouse and man that 50% of normal ZAP-70 activity is well tolerated, whereas an absence of activity leads to severe immunodeficiency. Activity between 0% and 50%, on the other hand, is often associated with unusual combinations of immunodeficiency and autoimmunity.[10–15] Quantitative differences in TCR signalling are also known to play a central role in establishing the correct repertoire of circulating T cells:[16] TCRs with high avidity for self-peptide/MHC trigger thymocyte negative selection, TCRs with intermediate avidity trigger positive selection, and TCRs with avidity that is too low fail to support thymocyte survival and maturation. Consistent with a quantitative model for signalling negative versus positive selection, strongly self-reactive TCRs have been shown to escape negative selection and instead undergo positive selection in mice with an amino acid substitution in the SH2 domain of ZAP-70.[11] By contrast, the catalytic domain mutant described here can still partially transduce TCR signals, yet does not allow appreciable numbers of T cells to mature.

Materials and methods

Mice

Zap70^{mrt} (MGI:3614790) was derived from a C57BL/6 male mouse treated intraperitoneally with 100 mg/kg *N*-ethyl-*N*-nitrosourea at 3-weekly intervals. Mutation mapping and DNA sequencing were performed as previously described.[17] *Zap70*^{tm1Weis} (*Zap70*$^{-/-}$, MGI:2176252), NOD.*H2*^{k} and 3A9 TCR transgenic (MGI:3512685) mice have been described previously,[3,18,19] and B10.Br/SgSnJ were obtained from The Jackson Laboratory. Mice were housed in a specific pathogen-free facility and all studies were conducted in compliance with protocols approved by the Animal Ethics and Experimentation Committee of The Australian National University.

Genotyping

The *mrtless* strain was maintained by breeding heterozygous carriers. Heterozygotes and homozygotes were identified by mutagenically separated PCR.[20] Primers used were 5′-CCGCAATGTTCTACTGGTCA-3′ (sense), 5′-GAAAGT TGATGCACTCTGGCGCGTATCA-3′ (antisense wild-type) and 5′-CACTGCGGCTGGAGAACTTCCTCAAGTTG ATGCACTCTGGCGCGTGCCG-3′ (antisense mutant). Amplification consisted of an initial 2 min at 94°; followed by 35 cycles of 20 seconds at 94°, 20 seconds at 62° and 30 seconds at 72°. The final cycle included an additional 10 min at 72°.

Flow cytometry

Blood was collected from the retro-orbital plexus in 10 μl of 500 mM EDTA. Red blood cells were lysed by addition of Tris–ammonium chloride, and the remaining cells were washed in PBS supplemented with 2% fetal calf serum. Suspensions of blood, thymus or spleen were stained with FITC-conjugated αCD4 (GK1.5), αCD45.1 (A20), αCD8 (53-6.7), αIgM (II/41) or αThy1.2 (53-2.1); phycoerythrin-conjugated αCD4 (GK1.5), αCD8 (53-6.7) or αIgD (IA6-2) (BD), or biotinylated αCD21 (C. Goodnow), αCD45.1 (A20), αCD45.2 (104), αCD5 (53-7.3), αCD69 (H1.2F3) or αTCR-β (H57-597) (Becton Dickinson, Franklin Lakes, NJ) followed by streptavidin Cy-chrome. Data were acquired on a FACSCalibur (Becton Dickinson) and analysed with FlowJo software (Treestar, Ashland, OR).

Western blotting

Thymocytes were sorted using a FACSVantage SE with DiVa Option (Becton Dickinson). Cell lysates were prepared in nonidet P-40 lysis buffer with sodium fluoride, PMSF, leupeptin hemisulphate, phenylarsine oxide, aprotinin and sodium orthovanadate. Samples were separated by SDS–PAGE using standard protocols and Western blotting was performed using a purified mouse monoclonal antibody against ZAP-70 (provided by A. Weiss), followed by goat anti-mouse horseradish peroxidase (ICN

Biomedicals, Costa Mesa, CA). Blots were visualized by chemiluminescence (Amersham, Little Chalfont, UK).

Proliferation assay

Ninety-six-well flat-bottom tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) were coated with a solution of PBS + 5 μg/ml αCD3 (2C11) for 75 min at 37°. Then, 5×10^5 CFSE-labelled splenocytes in a solution of complete RPMI-1640 + 1 μg/ml αCD28 (37.51) were added to each well, and incubated for 72 hr at 37° in 5% CO_2. After stimulation, cells were stained with CD4-peridinin chlorophyll protein-Cy5.5, CD8-phycoerythrin, CD69-phycoerythrin-Cy7 and Annexin V-allophycocyanin, and analysed by flow cytometry.

In vitro stimulation

The 96-well flat-bottom tissue culture plates (Corning Life Sciences, Corning, NY) were coated overnight at 4° with αCD3 (2C11) or αTCR (H57-597) in PBS, then washed and coated overnight with αCD28 (37.51). Thymocytes were resuspended in complete RPMI-1640, plated at 5×10^5 cells/well and incubated for 20 hr at 37° 5% CO_2. After stimulation, cells were stained for CD4, CD8 and CD69 and analysed by flow cytometry.

In vitro stimulation with hen egg lysozyme

Splenocytes were collected from a B10.Br mouse and irradiated (20 000 rads). Cells were plated on 96-well flat-bottom tissue culture plates and incubated for 6 hr at 37° 5% CO_2 in the presence of hen egg lysozyme (HEL) 46-61 peptide (1 μM). Thymocyte suspensions were then added to the plates and incubated for 15 hr at 37°, stained for CD4, CD8 and CD69, and analysed by flow cytometry.

In vivo stimulation with HEL

Mice on an $H2^{k/b}$ background expressing the 3A9 TCR transgene were injected intravenously with 1 mg HEL (Sigma, St Louis, MO) in PBS, or with PBS alone. Thymi were collected the following day, stained for CD4, CD8 and CD69, and analysed by flow cytometry. Where appropriate, B10.Br mice ($H2^{k/k}$) were used as wild-type controls.

Bone marrow chimeras

C57BL/6 CD45.1$^+$ recipient mice were given two doses of irradiation (500 rads each) separated by a 3-hr interval, then injected with 2×10^6 bone marrow cells through the tail vein. Recipient mice were killed for analysis 10 weeks later.

Results

Identification of a Zap70 missense mutation

In an investigation of genes required for lymphocyte development, blood samples from third-generation (G3) offspring of *N*-ethyl-*N*-nitrosourea-treated C57BL/6J sires were analysed by flow cytometry.[21] Within one pedigree, multiple individuals were found to lack CD4$^+$ and CD8$^+$ cells (Fig. 1a). Further breeding established that the phenotype, named *mrtless* (*mrt*), was inherited in an autosomal recessive manner.

A mapping cross was performed between C57BL/6J *mrtless* and NOD.$H2^k$ mice to determine the chromosomal location of the *mrtless* mutation. Of the 213 F_2 intercross (F_2IC) mice generated, 55 (25·8%) exhibited the *mrtless* phenotype, consistent with a fully penetrant recessive trait. Pooled DNA from the F_2IC was tested for linkage using a genome-wide panel of microsatellite markers, linking the T-cell-deficient phenotype to a marker on chromosome 1. DNA samples from individual F_2IC mice were then typed at additional microsatellite markers within the linked region, narrowing the mutation-containing interval to 13·6 Mb between D1Mit373 and D1Mit212 (Fig. 1b). Within this interval lay *Zap70*: a strong candidate given its known role in TCR signalling. *Zap70* cDNA from *mrtless* and C57BL/6 thymocytes was sequenced, and comparison revealed a T to C transition at nucleotide 1618 of *Zap70* (Fig. 1c) corresponding to a W504R amino acid substitution (Fig. 1d). W504 is located within the kinase domain of ZAP-70, adjacent to the catalytic activation loop (Fig. 1e), and is highly conserved among tyrosine kinases (Fig. 1g). Western blotting of sorted CD4$^+$ CD8$^+$ thymocyte lysates demonstrated that $Zap70^{mrt/mrt}$ mice did indeed express ZAP-70 protein, although at greatly diminished quantities compared with $Zap70^{+/+}$ controls. This reduction may be due to the structural instability of ZAP-70 introduced by the W504R substitution, which may also affect the catalytic activity of the residual protein. $Zap70^{mrt/+}$ CD4$^+$ CD8$^+$ thymocytes had a ~ 50% reduction in expression of ZAP-70 compared with wild-type (Fig. 1f), despite having no obvious impairment in T-cell proliferation, CD69 induction, or stimulation-induced phosphatidylserine expression (Fig. 1h). Nevertheless, heterozygosity for the $Zap70^{mrt}$ allele was sufficient to partially disrupt intracellular calcium flux in splenic T cells.[12,15]

To confirm that the block in T-cell development originated from T-cell-intrinsic defects in ZAP-70 function, mixed bone marrow chimeras were established to allow a direct comparison of CD45.2$^+$ cells (from $Zap70^{mrt/mrt}$ or $Zap70^{+/+}$ donors), and control CD45.1$^+$ cells developing within the same animal (Fig. 1i). Equal proportions of CD45.1$^+$ and CD45.2$^+$ CD4$^-$ CD8$^-$ thymocytes

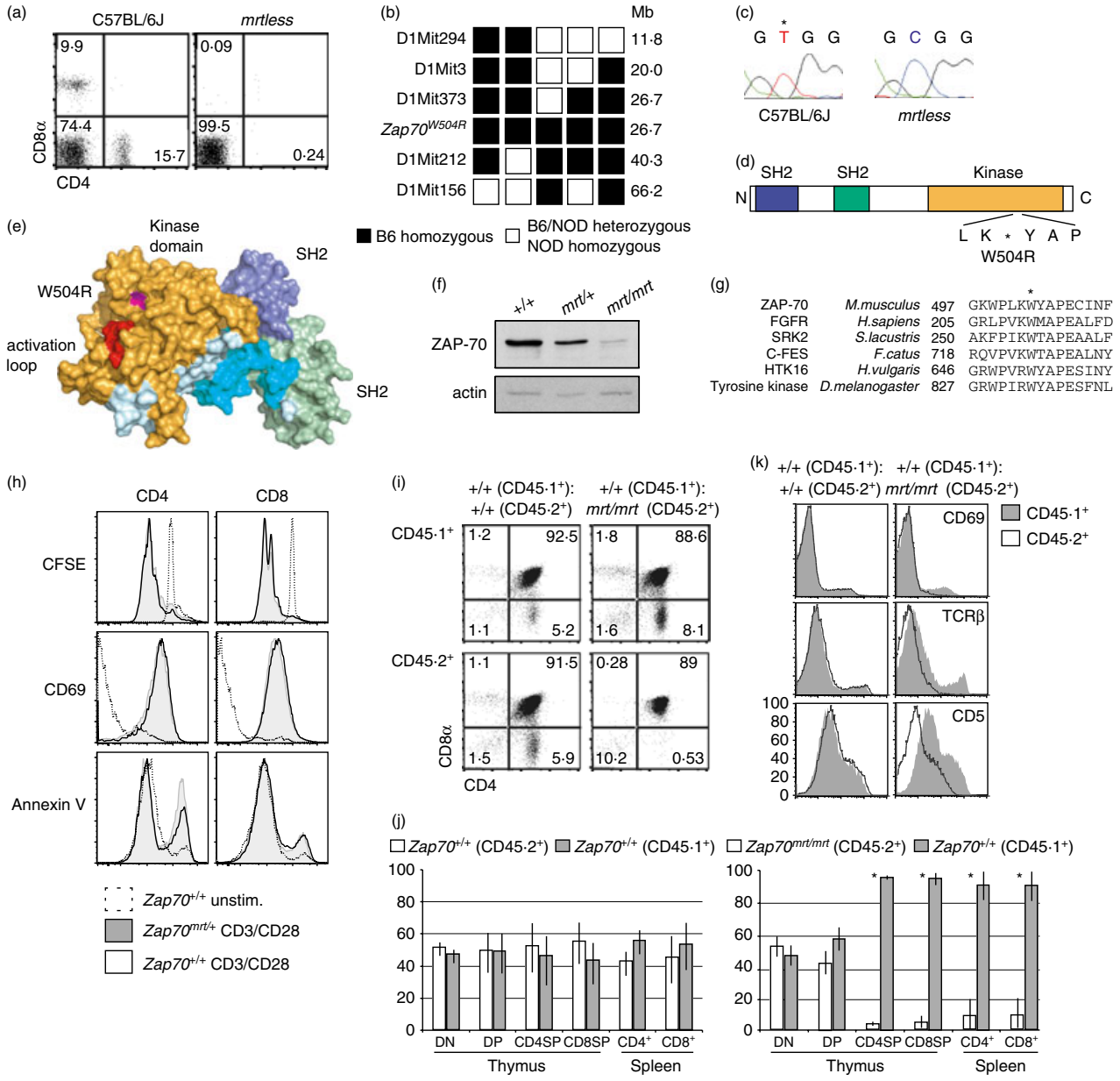

Figure 1. A loss-of-function missense variant of *Zap70*. (a) Flow cytometric analysis of peripheral blood lymphocytes from wild-type C57BL/6J and *mrtless* mice. (b) Individual F_2 intercross (F_2IC) mice with the *mrtless* phenotype were typed for the markers shown. Haplotypes of key recombinant F_2IC mice are displayed in columns. Black squares represent C57BL/6 homozygosity, while white squares represent C57BL/6-NOD heterozygosity or homozygosity. (c) Sequencing the cDNA of *mrtless* thymocytes identified a T to C transition at nucleotide 1618 of *Zap70*. (d) Schematic showing the *mrtless* mutation within the ZAP-70 protein. (e) Structural representation of ZAP-70 (PDB:2OZO), with W504 highlighted in magenta, and activation loop (CGNFGSVR) in red. (f) ZAP-70 Western blot on FACS-purified $CD4^+$ $CD8^+$ thymocyte lysates from mice of the indicated genotypes. (g) Cross-species kinase domain sequence alignment. Asterisk indicates the tryptophan mutated in the *mrtless* pedigree. (h) CFSE-labelled splenocytes from $Zap70^{+/+}$ and $Zap70^{mrt/+}$ mice were cultured in the presence or absence of anti-CD3 and anti-CD28 antibodies for 72 hr. Representative histograms ($n = 4$) show CFSE dilution and surface marker expression on $CD4^+$ and $CD8^+$ cells. (i,j) Sub-lethally irradiated $CD45.1^+$ mice were transplanted with a 1 : 1 mixture of either $Zap70^{+/+}$ and $Zap70^{+/+}$ bone marrow, or $Zap70^{+/+}$ and $Zap70^{mrt/mrt}$ bone marrow, and percentages of cells derived from each donor were established by flow cytometry. Bars in (j) indicate mean ± standard deviation, $n = 3$. (k) Histograms comparing CD69, T-cell receptor-β and CD5 expression upon $CD45.1^+$ cells (solid histogram) and $CD45.2^+$ cells (open histograms). Asterisk indicates statistical significance as determined by two-tailed Student's *t*-test ($P < 0{\cdot}05$). Data are representative of one (i–k), two (f, h) or more than two (a–c) independent experiments.

confirmed the establishment of balanced haematopoietic chimerism (Fig. 1j). In *Zap70*$^{mrt/mrt}$ mixed chimeras, however, there was a near complete absence of CD45.2$^+$ CD4$^+$ CD8$^-$, CD4$^-$ CD8$^+$ and peripheral CD4$^+$ or CD8$^+$ cells (Fig. 1i,j). This demonstrated that the *mrt-less* phenotype originated from a T-cell-intrinsic developmental arrest at the CD4^{++} CD8$^+$ stage, because very few *Zap70*$^{mrt/mrt}$ T cells were able to develop even under conditions where wild-type T cells could mature fully.

Expression of CD69, TCR-β and CD5 was also reduced on *Zap70*$^{mrt/mrt}$ thymocytes in mixed bone marrow chimeras (Fig. 1k). Few *Zap70*$^{mrt/mrt}$-derived thymocytes expressed CD69, whereas wild-type cells developing in the same thymus contained a normal subset of CD69$^+$ cells. Expression of TCR-β and CD5 was also diminished on *Zap70*$^{mrt/mrt}$ cells compared with wild-type cells in the same thymus, indicating that reduced induction of CD69, CD5 and TCR-β were all phenotypes intrinsic to *Zap70*$^{mrt/mrt}$ T cells.

Zap70$^{mrt/mrt}$ mice are phenotypically distinct to Zap70$^{-/-}$

Zap70$^{mrt/mrt}$ mice had normal numbers of CD4$^-$ CD8$^-$ and CD4$^+$ CD8$^+$ thymocytes, but an almost complete lack of CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ cells in the thymus and spleen: a phenotype equivalent to that observed in *Zap70*$^{-/-}$ mice (Fig. 2a,b). In response to pre-TCR and TCR signalling, CD4$^+$ CD8$^+$ thymocytes increase expression of CD3, CD5, CD69 and TCR-β before becoming a single-positive CD4$^+$ or CD8$^+$ cell.[22] *Zap70*$^{mrt/mrt}$ CD4$^+$ CD8$^+$ thymocytes showed weak expression of CD69, were devoid of CD3ε^{hi} and TCR-β^{hi} cells, and CD5 expression was markedly lower than *Zap70*$^{+/+}$ controls (Fig. 2c,d). Compared with *Zap70*$^{-/-}$ CD4$^+$ CD8$^+$ cells, however, CD5 and CD69 were still partially induced on *Zap70*$^{mrt/mrt}$ CD4$^+$ CD8$^+$ cells (Fig. 2c,d), confirming that these responses were ZAP-70-dependent and that the ZAP-70^{W504R} mutant kinase was partially capable of supporting this response.

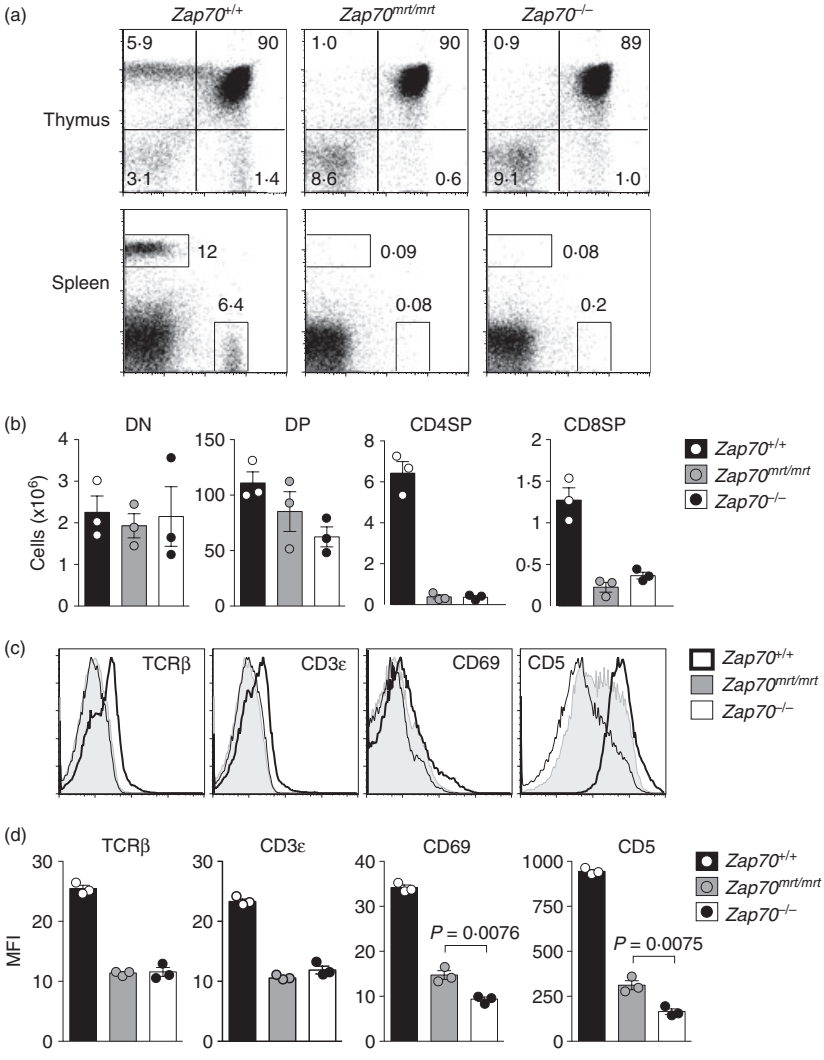

Figure 2. Evidence for partial ZAP-70 function in *Zap70*$^{mrt/mrt}$ mice. Flow cytometric analysis of thymic and splenic lymphocytes, with numbers indicating the percentages of total lymphocytes (a) or absolute numbers (b). (c, d) Comparison of surface marker expression upon CD4$^+$ CD8$^+$ thymocytes from *Zap70*$^{+/+}$ (thick black line), *Zap70*$^{mrt/mrt}$ (solid grey histogram) and *Zap70*$^{-/-}$ (thin black line) mice. Bars in (b) and (d) represent standard error of the mean, and *P*-values were calculated by two-tailed Student's *t*-test. Data are from one independent experiment ($n = 3$).

Zap70^{mrt} reduces, but does not abolish, responsiveness to TCR-derived signals

As T-cell development was intrinsically dysfunctional in *Zap70*$^{mrt/mrt}$ mice, it was of interest to assess the consequences of TCR/CD28 stimulation. Surprisingly, *Zap70*$^{mrt/mrt}$ CD4$^+$ CD8$^+$ thymocytes increased surface expression of CD69 in response to stimulation by αCD3 and αTCR antibodies *in vitro*, and did so to a greater extent than *Zap70*$^{-/-}$ cells (Fig. 3a,b). The response of *Zap70*$^{mrt/mrt}$ CD4$^+$ CD8$^+$ thymocytes was nevertheless lower than *Zap70*$^{+/+}$ controls at any given dose and combination of αCD3/αTCR and αCD28 (Fig. 3c,d). For instance, the

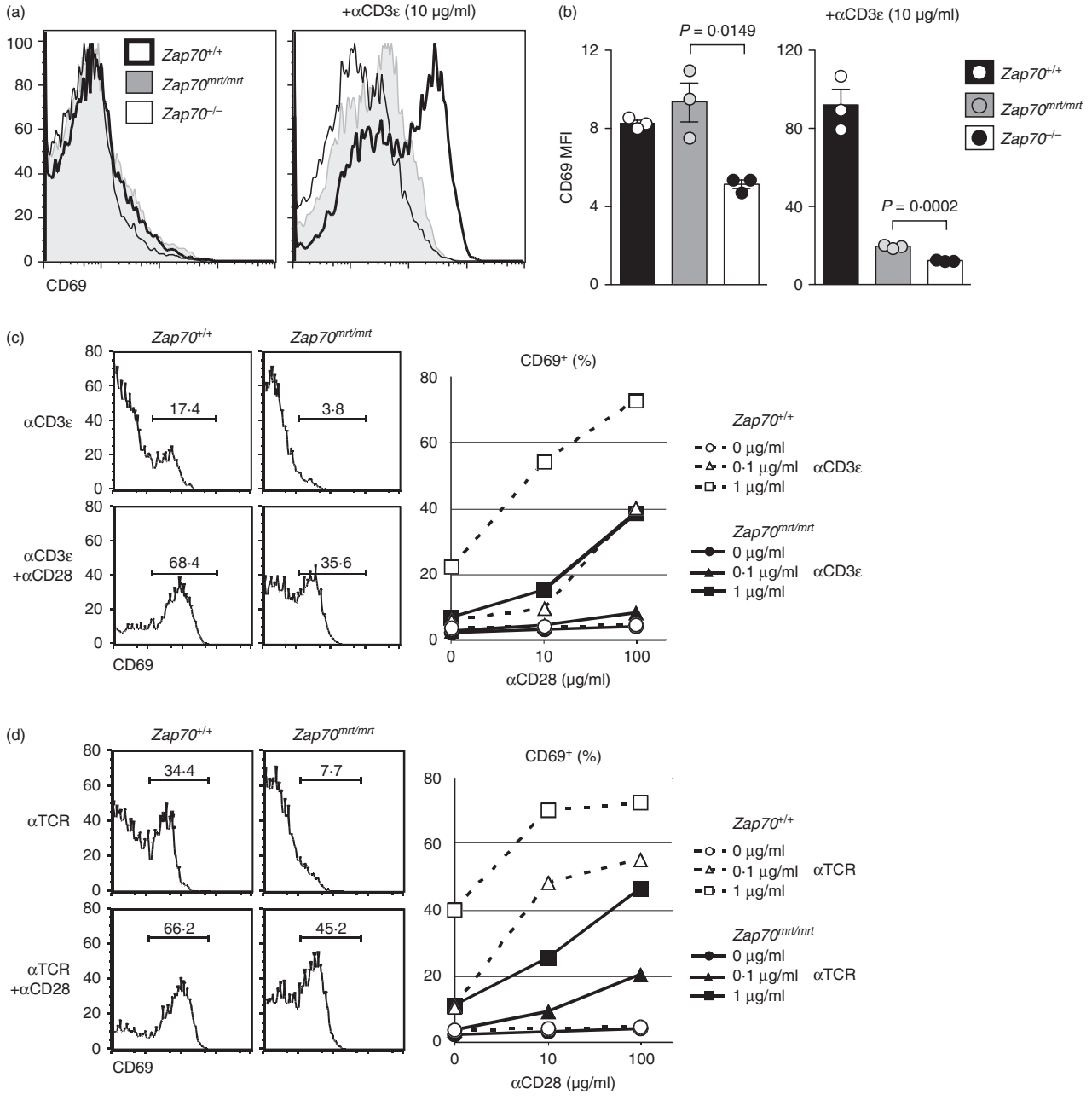

Figure 3. Shifted dose–response kinetics following T-cell receptor (TCR) stimulation *in vitro*. Thymocytes were stimulated for 20 hr in culture wells coated with anti-CD3 (a–c) or anti-TCR (d) in the presence or absence of anti-CD28, and the CD4$^+$ CD8$^+$ thymocyte response was examined by median CD69 expression (a) or by the percentage of cells that were CD69 positive (c,d). (a) Representative histogram of CD69 expression on *Zap70*$^{+/+}$ (filled), *Zap70*$^{mrt/mrt}$ (open, solid line) and *Zap70*$^{-/-}$ (open, broken line) after stimulation with 1 μg/ml anti-CD3 + 1 μg/ml anti-CD28. Bars in (b) represent standard error of the mean, and *P*-values were calculated by two-tailed Student's *t*-test. Data are representative of one (a, b) or two (c) independent experiments.

CD28 dose–response of *Zap70*$^{mrt/mrt}$ cells to 1 μg/ml αCD3 resembled that of *Zap70*$^{+/+}$ cells to 0·1 μg/ml αCD3 (Fig. 3c).

To determine the effects of the *mrtless* mutation upon antigen-specific responses, *Zap70*$^{mrt/mrt}$ mice were bred with mice transgenic for the α- and β-chains of the 3A9 TCR, which recognizes a HEL-derived peptide in the presence of H2^{k}.[19] 3A9 transgenic *Zap70*$^{mrt/mrt}$;*H2*$^{k/b}$ mice maintained the blockage in T-cell development at the CD4^{+} CD8^{+} stage, with very few CD4^{+} CD8^{-} or CD4^{-} CD8^{+} thymocytes present (Fig. 4a). Expression of TCR-β was higher and more uniform on CD4^{+} CD8^{+} thymocytes from TCR transgenic mice, and was reduced by approximately 50% in *Zap70*$^{mrt/mrt}$ thymocytes. CD5 expression was 80% lower on transgenic *Zap70*$^{mrt/mrt}$ CD4^{+} CD8^{+} thymocytes compared with controls (Fig. 4b). After a 15-hr stimulation with irradiated B10.Br (*H2*^{k}) splenocytes loaded with HEL antigen, CD69 was induced on both *Zap70*$^{mrt/mrt}$ and *Zap70*$^{mrt/+}$ thymocytes, although *Zap70*$^{mrt/mrt}$ cells had a mildly reduced response compared with *Zap70*$^{mrt/+}$ thymocytes (Fig. 4c).

Finally, to test antigen-specific responses *in vivo*, *H2*$^{k/k}$ TCR transgenic *Zap70*$^{mrt/mrt}$ or *Zap70*$^{mrt/+}$ mice and *Zap70*$^{+/+}$ controls were injected intraperitoneally with HEL in PBS, or with PBS alone. While HEL treatment had no measurable effect in non-transgenic *Zap70*$^{+/+}$ controls, it led to a strong induction of CD69 expression on *Zap70*$^{mrt/+}$ CD4^{+} CD8^{+} thymocytes. Consistent with the polyclonal dose–response shift, CD69 induction was 80% lower in *Zap70*$^{mrt/mrt}$ transgenic mice (Fig. 4d).

Discussion

Mutation of the human *ZAP70* gene (including those within the conserved catalytic domain), can abolish ZAP-70 activity and severely impair T-cell development.[4,6,7,23] Comparison with the published structure of the ZAP-70 kinase domain indicated that the mutated residue described here (ZAP-70^{W504R}) lies adjacent to the active site, and as such is likely to influence catalytic function.[24] A > 90% decrease in steady-state ZAP-70 protein in *Zap70*$^{mrt/mrt}$ mice was similar to that observed in mice

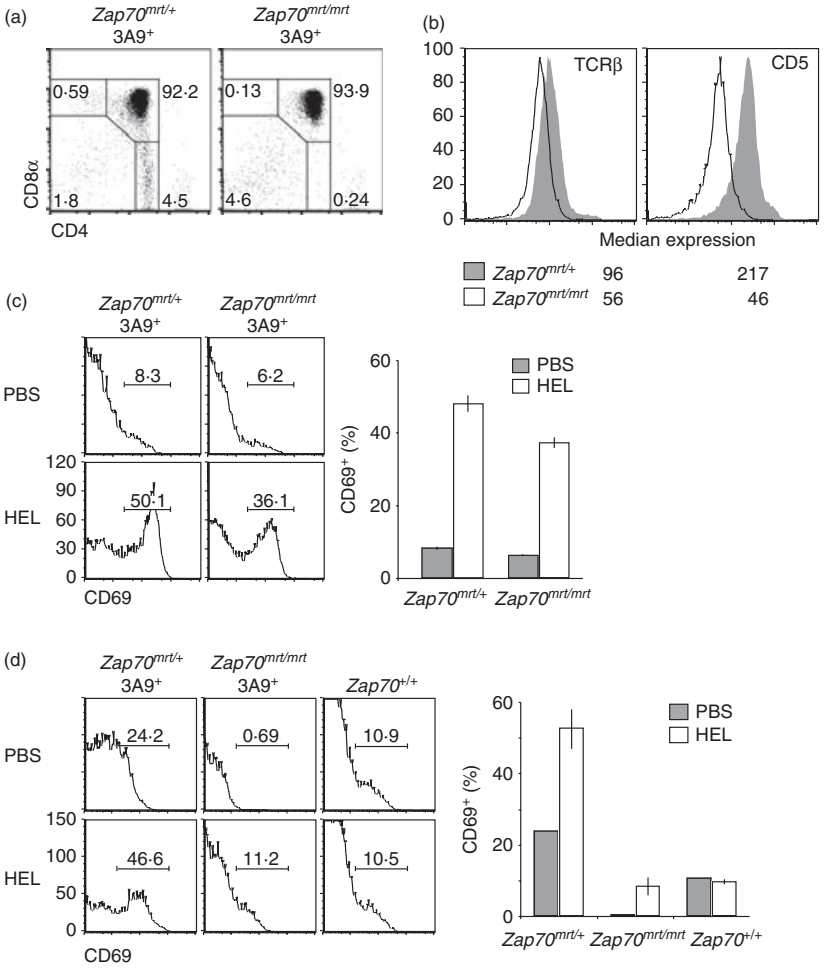

Figure 4. Effects of the *Zap70*$^{mrt/mrt}$ mutation on T cells with a defined T-cell receptor (TCR). (a) Flow cytometric analysis of thymocytes from 3A9 TCR transgenic mice (*H2*$^{k/b}$) carrying the *Zap70*^{mrt} mutation. (b) Representative histograms of TCR-β and CD5 expression on CD4^{+} CD8^{+} thymocytes from *Zap70*$^{mrt/+}$ (solid grey histograms) and *Zap70*$^{mrt/mrt}$ transgenic mice (unfilled histograms) (n = 2). (c) Thymocytes were stimulated *in vitro* for 15 hr with irradiated B10.Br (*H2*$^{k/k}$) splenocytes in the presence or absence of hen egg lysozyme (HEL) peptide, and flow cytometry was performed to measure CD69 induction on CD4^{+} CD8^{+} thymocytes. Representative histograms (left) and graphs (right) indicate mean ± standard deviation of CD69^{+} cells after no stimulation (grey bars, n = 2) or HEL stimulation (white bars, n = 2). (d) Mice were injected intraperitoneally with PBS, or 1 mg HEL in PBS, and thymi were collected 15 hr later. Flow cytometry was performed as in (c). Grey bars represent PBS control stimulation (n = 1), white bars represent HEL stimulation (n = 2). Data are from one independent experiment.

homozygous for the *Zap70*st (R464C) allele,[2] suggesting that catalytic site mutations can also destabilize ZAP-70 protein.

The fact that TCR-β, CD3ε and CD5 levels are diminished on *Zap70*$^{mrt/mrt}$ CD4$^+$ CD8$^+$ cells, even in the presence of TCR-$\alpha\beta$ transgenes, confirms that signalling through ZAP-70 is required to induce CD5 and maintain surface levels of TCR.[2] Reduced TCR expression on CD4$^+$ CD8$^+$ cells may indicate either a failure to increase translation of CD3 subunits, which normally occurs as thymocytes mature, or increased activity of the Lck/Cbl/SLAP pathway that down-regulates surface TCR. Indeed, over-expression of ZAP-70 in Jurkat cells competitively antagonizes Lck/Cbl/SLAP-mediated TCR down-regulation,[25] such that the reduction of ZAP-70 in *Zap70*$^{mrt/mrt}$ thymocytes may allow enhanced activity of the Cbl/SLAP pathway.

It was clear from comparison with *Zap70*$^{-/-}$ mice, however, that the *Zap70*^{mrt} allele retained a limited degree of function. CD5 and CD69 expression were both higher on *ex vivo* CD4$^+$ CD8$^+$ thymocytes, as was induction of CD69 expression in response to TCR stimulation. Differences such as these were reminiscent of alleles encoding phosphoinositide-3-kinase γ, where homozygous knockout animals show immunodeficiency and severe cardiac pathology, while kinase-dead mutants are only immunodeficient.[26] This difference has been ascribed to the ability of phosphoinositide-3-kinase γ to act as a macromolecular scaffold independently of its kinase activity. Similarly, while ZAP-70 kinase function and thymocyte maturation are presumably lost in both *Zap70*$^{-/-}$ and *Zap70*$^{mrt/mrt}$ animals, the retention of $< 10\%$ ZAP-70 protein with functional SH2-domains appears to be sufficient for partial induction of CD5 and CD69. Consistent with this, catalytic inactivation of ZAP-70 by a small molecule has revealed ZAP-70-dependent pathways that are entirely independent of its kinase activity, including regulatory T-cell suppression, and TCR-induced activation of the Rap1 GTPase.[27]

Given the ability of strong TCR agonists to induce CD69, and the evidence that a large proportion of newly rearranged TCRs have high avidity for self peptides,[28] it is surprising that very few CD4$^+$ CD8$^+$ cells in *Zap70*$^{mrt/mrt}$ mice express CD69 at levels associated with positive selection, and that almost no TCRs can compensate for the signalling defect to mature into single-positive T cells and accumulate in the periphery. This failure of TCR selection to compensate for the *Zap70*$^{mrt/mrt}$ mutation is all the more surprising when compared with mice homozygous for the *Zap70*^{skg} allele (W163C, in the proximal SH2 domain).[11] *Zap70*^{skg} homozygotes have a 50% decrease in the number of single positive thymocytes or peripheral cells, and animals develop lymphadenopathy, hypergammaglobulinemia and autoimmune arthritis.[11] When crossed with TCR transgenic strains, the *Zap70*$^{skg/skg}$ mutation decreases the efficiency of positive selection of the DO11 and HY TCRs approximately 90% and 96%, respectively, allowing male-specific TCRs with high self-avidity to escape negative selection and instead be positively selected.[11]

It is informative to consider how the disparate consequences of *Zap70*^{mrt} and *Zap70*^{skg} mutations fit with current models for TCR-induced positive versus negative selection. A simple TCR/ZAP-70 threshold model, where weak signals induce positive selection and strong signals induce negative selection, would predict that strongly self-reactive TCRs would compensate for the diminished ZAP-70 in *Zap70*$^{mrt/mrt}$ thymocytes. To fit the different effects of the *Zap70*^{mrt} and *Zap70*^{skg} mutations with this model, one might postulate that the *Zap70*^{mrt} mutation causes a quantitatively greater decrease in TCR signalling to all pathways. Hence, while many TCRs are able to compensate for the *Zap70*^{skg} mutation and support positive selection, presumably by recognizing self-peptides with higher avidity, TCRs with sufficiently high self-avidity to compensate for the *Zap70*^{mrt} mutation are rarely generated. In this view, it is surprising that CD69 and CD5 are still induced in *Zap70*$^{mrt/mrt}$ thymocytes, and that rare high-avidity cells do not homeostatically expand in the periphery of *Zap70*$^{mrt/mrt}$ mice.

Rather than quantitative thresholds for TCR/ZAP-70 signalling, an alternative explanation is that the *Zap70*$^{mrt/mrt}$ mutation causes qualitative and temporal changes in TCR signalling. These allow partial signalling to peptide exposure, which is sufficient for limited CD5 and CD69 induction in CD4$^+$ CD8$^+$ cells but insufficient to support the sustained signalling required for differentiation of CD4 and CD8 lineages.[29,30] Qualitative differences could involve changes in the substrate specificity of ZAP-70^{W504R} that prevent activation of particular signalling pathways. Alternatively, the temporal activity of the kinase may be altered such that it is unable to support positive selection due to increased turnover of the mutant protein, or because of saturating negative regulation by CD5, but can nevertheless deliver an acute burst of signalling for partial CD69 induction. One could also speculate that diminished amounts of ZAP-70 in *Zap70*^{mrt} but not *Zap70*^{skg} thymocytes allows enhanced inhibitory signalling through Lck-Cbl-SLAP. Since the Cbl-SLAP pathway inhibits positive selection through ZAP-70-dependent and -independent pathways,[25] enhancement of this pathway may compound diminished signalling through ZAP-70^{W504R} to bring about the failure of positive selection in *Zap70*$^{mrt/mrt}$ mice. A temporal sequence of TCR signalling in CD4$^+$ CD8$^+$ thymocytes has also been put forward as a mechanism for setting the bandwidth for TCR-positive selection, whereby TCR/ZAP-70 signals through the calcium/calcineurin/nuclear factor of activated T cells pathway are needed to enhance sensitivity of the CD4$^+$ CD8$^+$ cells to TCR/ZAP-70/extracellular signal-regulated kinase signalling, with both pathways necessary

for positive selection.[31] Failure to sustain TCR signalling through this sequence could explain the low induction of CD69 observed on *Zap70*$^{mrt/mrt}$ CD4$^+$ CD8$^+$ thymocytes compared with *Zap70*$^{-/-}$ thymocytes, yet the failure to induce CD69 to high levels. Which of these alternatives (if any) can explain the *Zap70*^{mrt} phenotype remains to be seen.

In conclusion, our data reveal a lower limit for ZAP-70 activity in thymic positive selection. This limit is clearly above zero, as emphasized by the different outcomes of TCR stimulation in *Zap70*$^{-/-}$ and *Zap70*$^{mrt/mrt}$ thymocytes. Although it is not yet clear if residual ZAP-70^{W504R} protein is catalytically active, or simply fills a cellular niche, it is nevertheless insufficient for the positive selection of thymocytes bearing low- and high-affinity TCRs alike. On the other hand, compound heterozygosity for ZAP-70^{W504R} and ZAP-70^{I367F} [12] or ZAP-70^{W504R} and ZAP-70^{A243V} [15] is sufficient for positive selection, yet not for peripheral activation, whereas mutation of only one allele is sufficient for both. Given the diversity of rare variants in TCR signalling, and their association with unique physiological outcomes,[13] the allelic series of *Zap70* variants will help define the narrow boundaries between immunity, severe immunodeficiency and immune dysregulation.

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Disclosures

The authors declare that they have no competing financial interests.

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