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Scalable signaling mediated by T cell antigen receptor–CD3 ITAMs ensures effective negative selection and prevents autoimmunity

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The T cell antigen receptor (TCR)-CD3 complex is unique in having ten cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). The physiological importance of this high TCR ITAM number is unclear. Here we generated 25 groups of mice expressing various combinations of wild-type and mutant ITAMs in TCR-CD3 complexes. Mice with fewer than seven wild-type CD3 ITAMs developed a lethal, multiorgan autoimmune disease caused by a breakdown in central rather than peripheral tolerance. Although there was a linear correlation between the number of wild-type CD3 ITAMs and T cell proliferation, cytokine production was unaffected by ITAM number. Thus, high ITAM number provides scalable signaling that can modulate proliferation yet ensure effective negative selection and prevention of autoimmunity.

Immunoreceptor tyrosine-based activation motifs (ITAMs; YXXL/I-$X_{6–8}$-YXXL/I, where 'Y' is tyrosine, 'X' is any amino acid, 'L/I' is leucine or isoleucine, and '-$X_{6–8}$-' is six to eight repeats of any amino acid) mediate signal transduction after tyrosine phosphorylation by forming a high-affinity docking site for the double Src homology 2 domain–containing protein tyrosine kinases (such as Zap70 and Syk)[1–3]. Adaptor molecules that contain ITAMs include the CD3ε (A000550), CD3γ (A000552), CD3δ (A000549) and CD3ζ (A000553) subunits of the T cell antigen receptor (TCR), the immunoglobulin-α and immunoglobulin-β chains of the B cell antigen receptor, and FcRγ and DAP12, which are included in several activating myeloid and natural killer cell receptors, such as immunoglobulin Fc receptors and NKG2D[4–7]. Such receptors contain many ITAMs, with the TCR complex having ten ITAMs distributed among its four CD3 subunits[3,8]. Precisely why the TCR requires so many ITAMs is not clear. It has been suggested that this high ITAM number could provide qualitative and/or quantitative contributions to T cell development and/or effector function[6,7]. However, in the absence of a system in which all CD3 ITAMs *in vivo* can be manipulated, this issue remains unresolved. It is also unknown how many ITAMs are needed to mediate the diverse events that are initiated by TCR signaling, such as tolerance induction, proliferation and cytokine production. In this study, we combined retrovirus-mediated stem cell gene transfer with multicistronic 2A peptide–linked retroviral vectors to facilitate functional analysis of all CD3 ITAMs *in vivo*[9]. This allowed us to generate 25 groups of mice expressing various combinations of wild-type and mutant ITAMs in TCR-CD3 complexes to assess the effect of a lower number of ITAMs on T cell development and function.

RESULTS

Profound autoimmunity in CD3-mutant mice

We first compared the spectrum of T cell development in recombination-activation gene 1–deficient (*Rag1*$^{-/-}$) mice reconstituted with retroviral vector–transduced donor bone marrow from mice lacking genes encoding CD3ε and CD3ζ (CD3εζ-KO mice) that also lacked CD3γ and CD3δ mRNA and protein because of gene silencing caused by the insertion of a phosphoglycerate kinase–neomycin-resistance cassette into the *Cd3e* locus[9–11] (**Supplementary Fig. 1a** online). The retroviral vectors used encoded wild-type or ITAM-mutant CD3 chains (**Supplementary Fig. 1b–d**). *Rag1*$^{-/-}$ recipient mice reconstituted with CD3εζ-KO bone marrow transduced with a vector encoding all four wild-type CD3 subunits (CD3δ^{WT}CD3γ^{WT}CD3ε^{WT} CD3ζ^{WT}; called 'CD3δγεζ^{WT}' here) had CD4 and CD8 expression patterns on thymocytes and splenocytes similar to those of *Rag1*$^{-/-}$ recipients of wild-type bone marrow transduced with empty vector (**Supplementary Fig. 1e**). The TCR surface expression on thymocytes

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Table 1 CD3 mutants

CD3δ	δ	M	δ	δ	M	M	δ	M	δ	δ	δ	δ	M	M	δ	M	δ	M	M	M	M	δ	M	δ	M
CD3γ	γ	γ	M	γ	M	γ	M	M	γ	γ	γ	M	γ	M	γ	γ	M	M	M	M	M	γ	γ	M	M
CD3ε	ε	ε	ε	M	ε	M	M	M	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε	M	M	M	M	M	M	M
CD3ζ	ζ	ζ	ζ	ζ	ζ	ζ	ζ	ζ	ζa	ζb	ζc	ζc	ζc	ζc	M	M	M	M	ζa	ζb	ζc	M	M	M	M
ITAMs	10	9	9	8	8	7	7	6	6	6	6	5	5	4	4	3	3	2	2	2	2	2	1	1	0
Experiments	15	3	3	3	2	2	2	4	2	2	2	2	2	2	12	2	2	9	2	2	2	4	3	5	3
Mice	73	15	15	15	9	10	10	20	10	9	10	10	10	10	61	9	10	45	10	10	10	20	15	10	15
Autoimmunity	–	–	–	–	–	–	–	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–

Wild-type and mutant CD3 combinations in retrogenic mice. Each Greek symbol indicates a wild-type CD3 chain, and 'M' indicates a tyrosine-to-phenylalanine substitution of both ITAM tyrosine residues in all ITAMs in that chain, except for the single CD3ζ mutants, for which 'ζa', 'ζb' and 'ζc' represent which ITAM is wild-type (the other two being a tyrosine-to-phenylalanine substitution); data below indicate number of intact ITAMs, number of experiments and mice analyzed, and development of autoimmunity (+) or no development of autoimmunity (–).

and splenocytes from these mice was also similar to that of cells from C57BL/6 mice (**Supplementary Fig. 1f**). In contrast, *Rag1*$^{-/-}$ recipient mice reconstituted with CD3εζ-KO bone marrow transduced with empty vector or with a vector encoding ITAM-mutant CD3 subunits (CD3δ^{M}CD3γ^{M}CD3ε^{M}CD3ζ^{M}; called 'CD3δγεζ^{M}' here) showed severely impaired T cell development, even though B cell reconstitution was normal; thus, these mice had a phenotype similar to that of unmanipulated CD3εζ-KO mice (**Supplementary Fig. 1e**). We then generated 23 additional retroviral vectors encoding various combinations of wild-type and mutant CD3 ITAMs for subsequent analysis (**Table 1**). Some combinations included mutant CD3ζ subunits that had only one wild-type ITAM (for example, in CD3ζa^{WT}ζbc^{M}, 'a' is the membrane-proximal ITAM, 'b' is the middle ITAM and 'c' is the membrane-distal ITAM).

Unexpectedly, mice with two wild-type ITAMs (CD3ε^{WT}CD3δγζ^{M}; called 'CD3ε^{WT}δγζ^{M}' here) or four wild-type ITAMs (CD3δγε^{WT}CD3ζ^{M}; called 'CD3δγε^{WT}ζ^{M}' here) became sick 5–6 weeks after bone marrow transfer and died within 1 week of the onset of signs that included lethargy, hunched posture, ruffled coat, dehydration and squinting but limited weight loss (**Fig. 1a**). This early and rapid disease morbidity and mortality was particularly notable, as T cells were not detectable in the periphery until approximately 3 weeks after bone marrow transfer, and complete T cell reconstitution required approximately 8 weeks. *Rag1*$^{-/-}$ recipients of CD3ζ-KO bone marrow transduced with CD3ζ^{M} retrovirus had an autoimmune disease similar to that noted when CD3εζ-KO donor bone marrow was used, but recipients of CD3ζ-KO bone marrow transduced with CD3ζ^{WT} retrovirus did not (**Supplementary Fig. 2** online). This finding indicated that ITAM number rather than the retroviral vector transduced (single versus 2A-linked multicistronic) and/or bone marrow donor (CD3ζ-KO versus CD3εζ-KO) was responsible for the disease phenotype (data not shown). This disease could also be adoptively transferred with splenocytes from sick CD3δγε^{WT}ζ^{M} and CD3ε^{WT}δγζ^{M} bone marrow chimeras but not those from healthy CD3δγεζ^{WT} bone marrow chimeras (**Fig. 1b**).

The development of autoimmunity in the CD3δγε^{WT}ζ^{M} bone marrow chimeras was also notable because CD3ζ-KO mice expressing a 'Tg1-6^{M}' mutant transgene in which sequence encoding each ITAM tyrosine was replaced with sequence encoding phenylalanine (and thus having the same number of wild-type ITAMs as the CD3δγε^{WT}ζ^{M} bone marrow chimeras) develop only very mild indications of inflammatory disease in old age[12–14]. This difference was not due to our experimental approach, as *Rag1*$^{-/-}$ recipients of CD3ζ-KO Tg1-6^{M}-transgenic bone marrow transduced with empty vector also failed to develop disease (**Supplementary Fig. 2a**). Notably, thymocytes and splenocytes from CD3ζ-KO Tg1-6^{M}-transgenic mice had much higher TCR surface expression than that of C57BL/6 mice or mice with retroviral transgenic ('retrogenic') expression of CD3δγεζ^{WT} or CD3δγεζ^{M}, which may have masked and/or compensated for any signaling deficiencies during negative selection (**Supplementary Fig. 2b–d**). It is noteworthy that T cells in CD3ζη-KO mice, which have lower expression of surface TCR, overtly react with self major histocompatibility complex (MHC) once normal TCR expression is restored[15].

Histological examination of the sick mice showed a broad spectrum of tissues affected (**Supplementary Results** and **Tables 1,2** online). The most severe pathology was in the lungs, liver and gastrointestinal tract (**Fig. 1c**). Death was probably due to multiorgan inflammatory processes characterized by colitis, and vasculitis and perivasculitis in the lung and liver.

Subsequent analysis of all 25 groups of mice expressing wild-type or mutant CD3 showed that 13 groups developed very similar signs and had similar lesions by histological examination (**Table 1** and **Supplementary Table 1**). These groups had two to six wild-type ITAMs. Notably, only two of the four groups with six wild-type ITAMs became sick, which suggested that the type of ITAM present can also affect disease outcome. In general, the kinetics of disease onset slowed with fewer wild-type ITAMs, which suggested a direct correlation between CD3 signal strength (proportional to functional wild-type ITAM number) and disease severity and/or time to disease onset (**Fig. 1d** and **Supplementary Fig. 3c** online). Disease penetrance was 100% in these 13 groups of mice. Immunofluorescence analysis of lung, liver, intestine and kidney showed substantial CD4^{+} T cell infiltration and macrophage activation but more limited infiltration of CD8^{+} T cells and CD19^{+} B cells (**Fig. 1e** and **Supplementary Fig. 4** online). We confirmed an absolute requirement for CD4^{+} T cells in the initiation and progression of autoimmunity by adoptive transfer of disease with purified CD3δγε^{WT}ζ^{M} CD4^{+} T cells but not CD8^{+} T cells or CD3δγεζ^{WT} CD4^{+} wild-type control cells (**Supplementary Fig. 5** online).

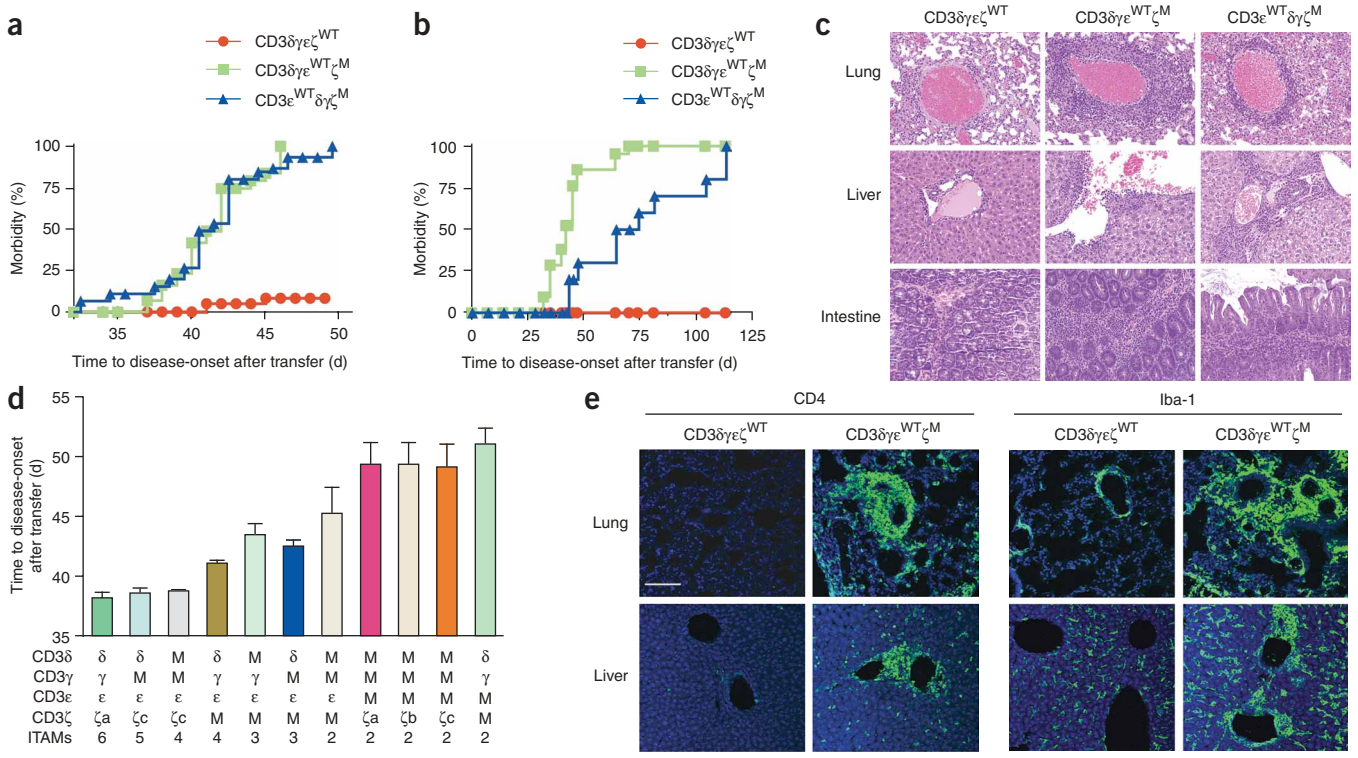
Many autoimmune diseases are characterized by the development of autoantibodies to a variety of host antigens. We used autoantigen microarrays to compare the reactivity profiles of serum from CD3-wild-type and CD3-mutant retrogenic mice[16]. Hierarchical clustering and significance analysis of microarrays[17] showed that a small proportion of serum from CD3-mutant mice had statistically significant autoantibody reactivity patterns. Whereas serum from most of the CD3δγε^{WT}ζ^{M} mice did not show an antigen-binding pattern that was significantly different from that of CD3-wild-type controls, except for some borderline positive reactivities, serum from CD3δγζ^{WT}ε^{M} and CD3ε^{WT}δγζ^{M} mice clustered together with positive control serum from mice with pristane-induced systemic lupus erythematosus (**Fig. 1f** and **Supplementary Fig. 6** and **Table 3** online). Notably, there were both overlapping reactivities (for example, to single-stranded and

double-stranded DNA) and unique reactivities (for example, to flax flagellen, a peptide of bacterial flagella). In addition, serum from one of the two CD3δγεζa^{WT}ζbc^{M} mice developed vasculitis-associated autoantibodies directed against neutrophil antigens (**Supplementary Fig. 6**). It is unclear why only certain CD3-mutant mice developed autoantibodies. The detection of autoantibodies in CD3δγζ^{WT}ε^{M} mice was notable, given that these mice failed to develop symptomatic disease in the time frame of this study (50 d), and may suggest the existence of more chronic forms of autoimmunity in mice with six to nine functional CD3 ITAMs.

High ITAM number required for T cell development

To identify the defects that led to the severe autoimmune disease of the ITAM-mutant mice, we first assessed the efficiency of T cell development and establishment of a peripheral T cell pool in mice expressing each of the 25 CD3 ITAM combinations. Another goal of this analysis was to calculate the number and type of ITAMs required for various T cell–developmental parameters (such as thymocyte number, percentage of CD4 single-positive thymocytes, splenic T cell number and so on). We initially generated 446 mice having at least 40% reconstitution with green fluorescent protein–positive (GFP$^+$) splenocytes in 15 experiments (**Table 1**, **Fig. 2** and **Supplementary Fig. 7** online). Each parameter examined required a specific threshold number of wild-type ITAMs (such as seven ITAMs for thymocyte number, four for splenic T cell number, two for CD4$^+$ cell/CD8$^+$ cell ratio), rather than a linear relationship correlating with ITAM numbers.

In general, mice expressing seven to nine wild-type CD3 ITAMs had a pattern of T cell development and peripheral T cell seeding similar to that of wild-type control mice (**Fig. 2**). There were differences in the total number of splenic T cells in each group, and the low error and



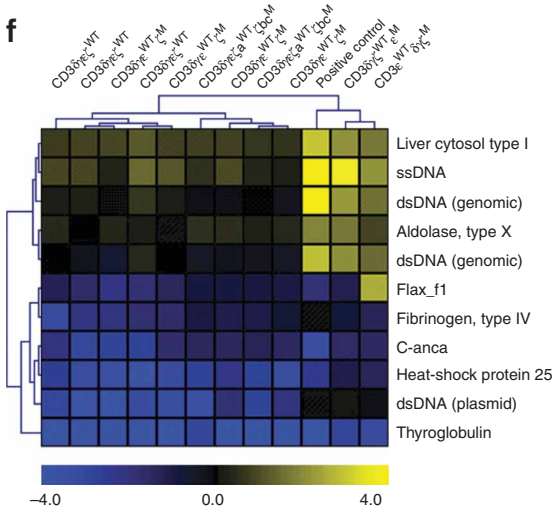


Figure 1 Profound autoimmunity in CD3-mutant mice. (**a**) Time of onset of sickness in *Rag1*$^{-/-}$ mice after transfer of bone marrow from CD3δγεζ^{WT} mice ($n = 60$ recipient mice), CD3δγε^{WT}ζ^{M} mice ($n = 45$ recipient mice) or CD3ε^{WT}δγζ^{M} mice ($n = 43$ recipient mice). (**b**) Time of onset of sickness in *Rag1*$^{-/-}$ recipients of 8×10^6 whole splenocytes from spleens collected from CD3δγεζ^{WT} mice ($n = 21$ recipient mice), CD3δγε^{WT}ζ^{M} mice ($n = 21$ recipient mice) or CD3ε^{WT}δγζ^{M} mice ($n = 10$ recipient mice) after the mutant mice had become sick. (**c**) Hematoxylin and eosin staining of formalin-fixed, paraffin-embedded sections of organs from the mice in **a**. (**d**) Time to onset of sickness (mean and s.e.m.) after bone marrow transfer (designations below graph as in **Table 1**). (**e**) Immunofluorescence staining and DAPI counterstaining of formalin-fixed cryostat sections to detect CD4$^+$ T cells and Iba-1$^+$ activated macrophages. (**f**) Reactivity profiles of serum from CD3-wild-type and CD3-mutant mice compared by autoantigen array. Analysis of microarrays[17] identified antigens with statistically significant differences in array reactivity for CD3-wild-type and CD3-mutant mice (**Supplementary Table 3**). Serum from mice with pristane-induced systemic lupus erythematosus serves as a positive control. ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Flax_f1, flax flagellen; C-anca, antibody to neutrophil cytoplasmic antigen. Data are the mean of eight to ten separate experiments (**a**) or two to three separate experiments (**b**) or are representative of five to ten mice per group (**c**), two to four experiments with 9–20 mice (**d**), two experiments with two mice per group (**e**) or three independent experiments with 33 serum samples from six groups (**f**).

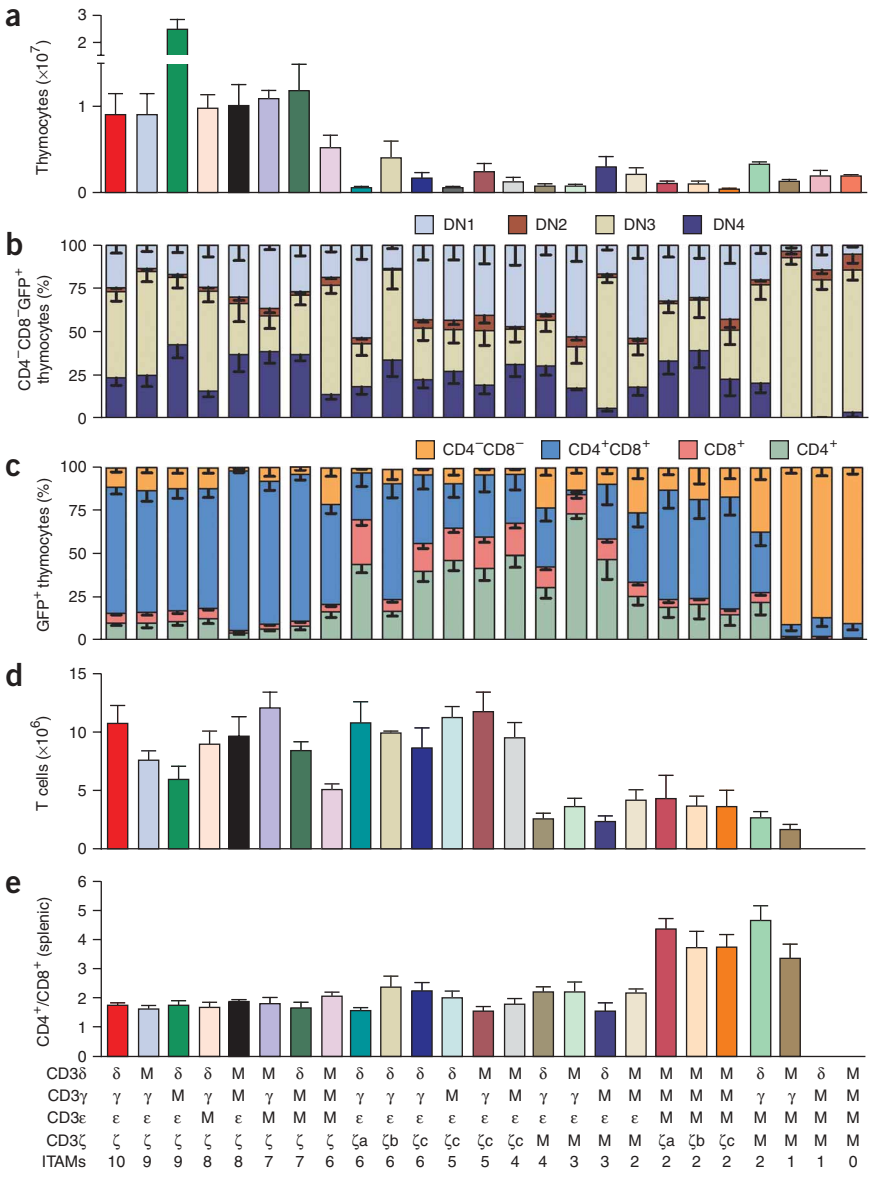


Figure 2 A specific number of ITAMs is required for each T cell developmental parameter. Analysis of *Rag1*$^{-/-}$ recipient (retrogenic) mice 5–8 weeks after transfer of transduced CD3εζ-KO bone marrow. (**a**) Total number of thymocytes in each group. (**b**) Flow cytometry of thymocytes stained for CD4, CD8, CD25 and CD44 and gated on live GFP$^+$CD4$^-$CD8$^-$ cells, with a quadrant gate set to obtain percent cells at each double-negative stage (DN1–DN4). (**c**) Flow cytometry of thymocytes stained for CD4 and CD8 and gated on live GFP$^+$ cells, with a quadrant gate set to obtain percent of each population. (**d**) Flow cytometry of percent live GFP$^+$TCRβ$^+$ cells to assess splenic T cell numbers. (**e**) Ratio of CD4$^+$ splenocytes to CD8$^+$ splenocytes for cells analyzed as described in **d**. Data are the mean and s.e.m. of 9–40 mice from two to eight separate experiments per group.

large number of mice examined indicated that these findings were not due to experimental variation but instead were due to subtle differences imposed by the individual ITAMs. The most notable difference among the groups with seven to nine wild-type CD3 ITAMs was that CD3δεζ^{WT}γ^{M} mice had 2.5-fold higher thymocyte numbers than did CD3δγεζ^{WT} mice (**Fig. 2a**). CD3γεζ^{WT}δ^{M} mice did not have this larger number of thymocytes, which suggested that small differences in ITAM sequence and/or location can influence TCR signaling and thymocyte development.

In contrast, we noted substantially lower total thymocyte numbers in mice with six or fewer wild-type ITAMs, which suggested alterations in selection, survival and/or egress (**Fig. 2a**). Pre-TCR signaling, which mediates the transition from the double-negative 3 stage to the double-negative 4 stage, seemed essentially normal in mice with two or more wild-type ITAMs (**Fig. 2b** and **Supplementary Fig. 8a** online). These data also show that effective pre-TCR signaling can occur with only two functional ITAMs, which supports the idea that nominal tonic signaling induced by expression of rearranged TCRβ–pre-TCRα complexes is sufficient to mediate transition from the double-negative 3 stage to the double-negative 4 stage[18]. Upregulation of CD5 on single-positive thymocytes, a marker of positive selection[19], for the most part correlated with thymic output, such that mice with six or more functional ITAMs that did not develop autoimmunity had normal CD5 expression, whereas mice with six or fewer functional ITAMs that did develop disease had low CD5 upregulation (**Supplementary Fig. 8b**,**c**). For unknown reasons, mice with very few functional ITAMs seemed to have higher CD5 expression than that of other groups. Many of the groups that developed autoimmunity (three to six wild-type ITAMs) also had a much lower percentage of double-positive thymocytes and an associated increase in the ratio of single-positive cells to double-positive cells (**Fig. 2c** and **Supplementary Fig. 7c**). Although some of the decrease in double-positive populations could have been due to disease-induced release of steroid hormones, much of this analysis was done before disease onset. Furthermore, there were two groups of mice with six wild-type ITAMs (CD3ζ^{WT}δγε^{M} and CD3δγεζb^{WT}ζac^{M}) that had fewer total thymocyte numbers but did not develop the autoimmune disease or pathology.

Thymocyte cellularity did not always correlate with the size of the peripheral T cell pool, as six groups had a low number of thymocytes but essentially normal splenic T cell numbers (**Fig. 2d**). Discrepancies between thymocyte and splenic T cell numbers did not correlate with the ratio of single-positive cells to double-positive cells or disease state and thus are more likely a consequence of homeostatic proliferation that resulted in 'filling up' of the peripheral T cell space. Notably, these six groups were the only groups with an intact CD3ε ITAM and at least one intact CD3ζ ITAM, which suggested that these ITAMs may be key in T cell homeostasis. Whereas the proportion of αβ T cells in the spleen varied considerably among the CD3-mutant groups, the percentage of γδ T cells in the spleen and Peyer's patch of CD3-mutant groups was the same or higher than that in mice with wild-type CD3 over a broad ITAM range, which suggested that relatively low TCR signal strength may be sufficient for normal development and homeostasis of γδ T cells (**Supplementary Fig. 9** online). CD3δγε^{WT}ζ^{M}

mice, which developed rapid autoimmunity, had many more γδ T cells, especially in the Peyer's patches. Whether this is a consequence of the autoimmune status of these mice remains to be determined.

TCR surface expression on peripheral T cells and TCR variable β-chain (V_β) use were unaltered between wild-type mice and mutant mice that developed disease (**Supplementary Fig. 7d**, **8d**). The $CD4^+$ T cell/$CD8^+$ T cell ratio was normal (2:1) in the spleens of mice with three or more wild-type ITAMs but was much higher (approximately 4:1) in mice with fewer than three wild-type ITAMs (**Fig. 2e**). The one exception was $CD3\varepsilon^{WT}\delta\gamma\zeta^{M}$ mice, which had a normal $CD4^+$ cell/$CD8^+$ cell ratio; this suggested that a functional CD3ε ITAM alone was uniquely sufficient to control this balance. Finally, the two groups with only one functional ITAM had almost no double-negative 4, double-positive or single-positive thymocytes. Of these two groups, mice with only a wild-type CD3δ ITAM had no splenic T cells, but 40% of mice with only a wild-type CD3γ ITAM nevertheless had detectable splenic $CD4^+$ T cell populations (**Fig. 2d**).

The data reported above collectively demonstrate that each parameter examined required a specific threshold of wild-type ITAMs. It is evident that the number (quantitative parameter) rather than the type (qualitative parameter) of wild-type ITAMs was the main defining factor. However, we noted a secondary qualitative influence of certain CD3 ITAMs, most notable for CD3γ verses CD3δ. Thus, differential recruitment of 'downstream' signaling molecules and/or location in the TCR-CD3 complex may also influence T cell development.

Low ITAM number and T cell function

We next assessed the number of CD3 ITAMs required for peripheral T cell proliferation and cytokine production in response to the 'superantigen' staphylococcal enterotoxin B (there were no differences in the percentage of $V_\beta7^+$ or $V_\beta8^+$ T cells reactive to staphylococcal enterotoxin B among any of the groups examined; **Supplementary Fig. 8d** and data not shown). In contrast to the discrete number of wild-type ITAMs required for each of the developmental parameters examined above, there was a precise linear relationship between the number of CD3-wild-type ITAMs and T cell–proliferative capacity (**Fig. 3a**,**b** and **Supplementary Fig. 10** online). The groups that were most displaced from the linear regression line all had a single CD3γ or CD3δ wild-type ITAM (**Supplementary Fig. 10a**), which suggested a unique modulatory influence for these ITAMs on T cell proliferation.

In contrast, the presence of fewer wild-type CD3 ITAMs had no significant effect on cytokine production (**Fig. 3c–g**). However, the type of CD3 ITAM present did seem to have some modulatory effect on cytokine production. T cells from mice that developed rapid autoimmunity produced more of the proinflammatory cytokine interferon-γ (IFN-γ) than did those from wild-type mice. Notably, T cells with only a single wild-type CD3γ ITAM produced almost the same amount of interleukin 2 (IL-2), IL-4 and IFN-γ as wild-type T cells did (**Fig. 3c–e**) and produced nearly twice as much IL-5 and IL-10 as wild-type T cells did (**Fig. 3f**,**g**). These data indicate that a low threshold number of CD3 ITAMs is required for cytokine production.

Although V_β use was unaltered by the number of wild-type CD3 ITAMs, it is possible that there may have been alterations in the TCR

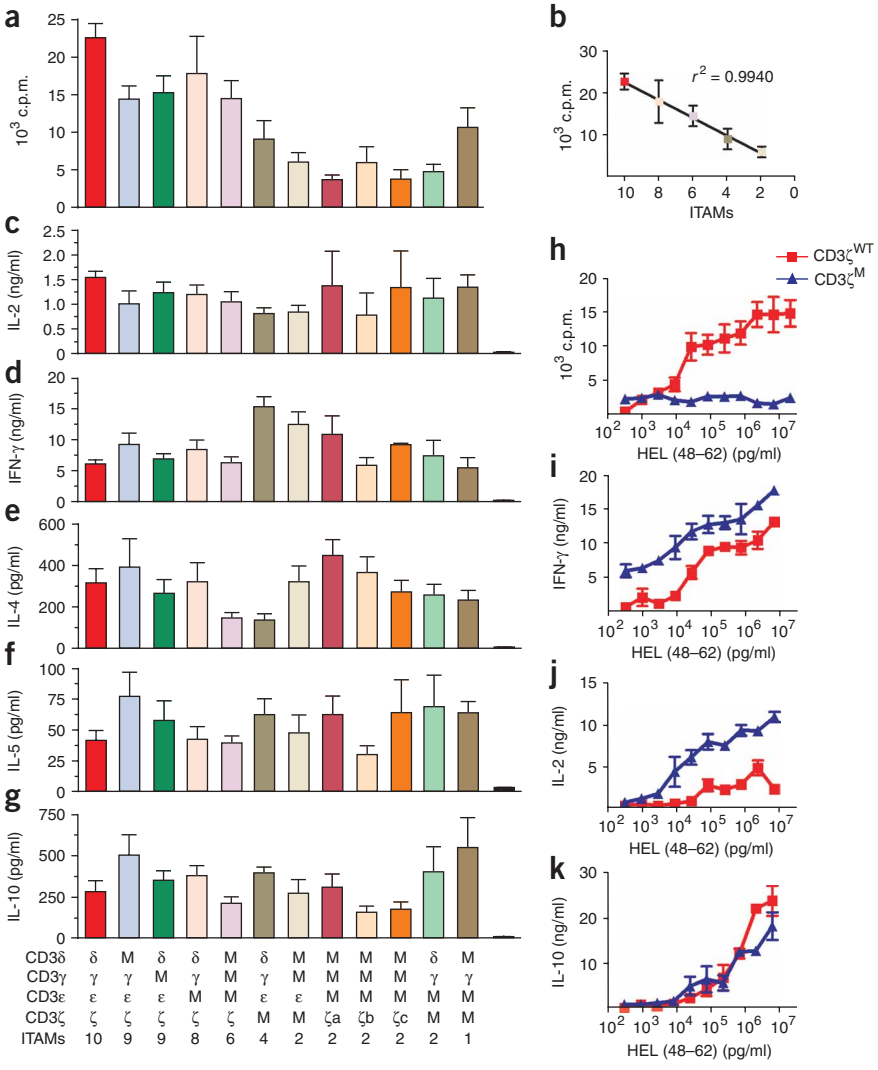


Figure 3 Cells from CD3-mutant mice proliferate less but have normal cytokine production. (**a**–**g**) Analysis of cells from *Rag1*$^{-/-}$ recipient (retrogenic) mice 5–8 weeks after transfer of CD3εζ-KO bone marrow; splenic T cells (2×10^5) were purified by magnetic-activated cell sorting and stimulated for 48 h with staphylococcal enterotoxin B (30 μg/ml) plus irradiated C57BL/6 splenocytes (5×10^5). (**a**,**b**) Cells pulsed for a further 24 h with [^{3}H]thymidine. Data in **b** are a linear regression of the data in **a**. (**c**–**g**) Analysis of cytokines in 50 μl of the supernatant immediately after stimulation; far right bars, limit of detection or media control. Data are the mean and s.e.m. of four to ten separate experiments. (**h**–**k**) Analysis of cells from *Rag1*$^{-/-}$ recipient (retrogenic) mice 8 weeks after transfer of transduced CD3ζ-KO 3A9 TCR–transgenic bone marrow: splenic T cells (2×10^5) purified by magnetic-activated cell sorting were stimulated for 48 h with various concentrations of HEL (48–62) plus irradiated B10.BR splenocytes (5×10^5). (**h**) Cells pulsed for a further 24 h with [^{3}H]thymidine. (**i**–**k**) Analysis of cytokines in 50 μl of the supernatant immediately after stimulation. Data are the mean ± s.e.m. of two separate experiments.

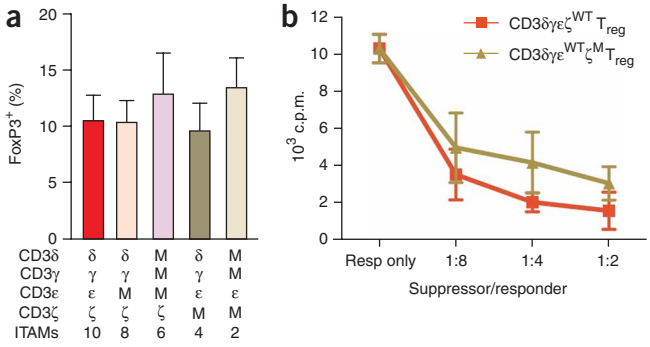


Figure 4 Normal proportion and function of T_{reg} cells in CD3-mutant mice. (**a**) Staining of CD4 and Foxp3 in splenocytes from retrogenic mice generated by retrovirus-mediated stem cell gene transfer, assessed 5–6 weeks after transplant with gating on $CD4^+$ lymphocytes. Data are the mean and s.e.m. of two separate experiments. (**b**) Suppressive activity of spleen and lymph node cells from the mice in **a** 6 weeks after transplant, sorted based on a $GFP^+CD4^+CD45RB^{lo}CD25^+$ profile for T_{reg} cells (suppressor) and a $GFP^+CD4^+CD45RB^{hi}CD25^-$ profile for naive T cells (responder (Resp)), mixed at various ratios (horizontal axis) and stimulated with antibody to CD3 plus irradiated C57BL/6J splenocytes. Data are the mean ± s.e.m. of two separate experiments.

repertoire as a consequence of selection of peptide-MHC recognition with higher affinity to compensate for fewer wild-type ITAMs[20]. To address that possibility, we transduced bone marrow from CD3ζ-KO mice expressing a transgene encoding the 3A9 TCR (specific for the peptide hen egg lysozyme amino acids 48–62 (HEL (48–62)) and H-$2A^k$ restricted) with either $CD3\zeta^{WT}$ or $CD3\zeta^M$ retroviral vectors. Consistent with their non–TCR-transgenic counterparts, they had fewer thymic and splenic $CD3\zeta^M$ 3A9 T cells than the wild-type controls had (**Fig. 2a,d**, and **Supplementary Fig. 11a,b** online). However, we found no disease in these mice, which may have been due in part to the high $CD4^+$ splenic T cell/$CD8^+$ splenic T cell ratio induced by the MHC class II–restricted TCR transgene (data not shown and **Supplementary Fig. 11c**). $CD3\zeta^{WT}$ 3A9 T cells but not $CD3\zeta^M$ 3A9 T cells proliferated in response to peptide stimulation (**Fig. 3h**). In contrast, $CD3\zeta^M$ 3A9 T cells secreted much more IL-2 and IFN-γ than $CD3\zeta^{WT}$ 3A9 T cells did, although some secretion of the former was due to a lack of proliferation-driven autocrine consumption (**Fig. 3i,j**). Production of IL-10 by $CD3\zeta^{WT}$ and $CD3\zeta^M$ 3A9 T cells was similar, which indicated that having 60% fewer functional ITAMs had little effect on cytokine production (**Fig. 3k**). Thus, whereas lower CD3 signal strength limited T cell–proliferative capacity, the ability to produce a wide array of cytokines remained mostly intact.

Lymphopenia is not required for autoimmune disease

To determine what caused disease in the CD3-mutant mice, we first assessed the contribution of T cell lymphopenia. Although many of the groups that developed this disease were lymphopenic, several factors suggested an etiology distinct from lymphopenia-driven diseases (**Supplementary Results** and **Supplementary Fig. 12** online). Despite those differences, we addressed this issue directly by generating mixed retrogenic chimeras by reconstituting *Rag1*$^{-/-}$ mice with various combination of CD3εζ-KO bone marrow transduced with $CD3\delta\gamma\varepsilon^{WT}\zeta^M$, $CD3\varepsilon\delta\gamma\zeta^{WT}$ or empty vector (**Supplementary Fig. 13a** online). The reconstitution, T cell development and disease onset of wild-type $CD3\varepsilon\delta\gamma\zeta^{WT}$ and mutant $CD3\delta\gamma\varepsilon^{WT}\zeta^M$ control groups was similar to that in previous experiments. CD3εζ-KO bone marrow transduced with empty vector and injected together affected the percentage of 'marked' bone marrow and B cells but had no effect on T cell reconstitution or disease score (**Supplementary Fig. 13a–c**). Mice injected with equal proportions of bone marrow transduced with $CD3\varepsilon\delta\gamma\zeta^{WT}$ and bone marrow transduced with $CD3\delta\gamma\varepsilon^{WT}\zeta^M$ had T cell populations similar in size to those of mice injected with bone marrow transduced with $CD3\varepsilon\delta\gamma\zeta^{WT}$ alone or with a 50:50 mixture of bone marrow transduced with $CD3\varepsilon\delta\gamma\zeta^{WT}$ and bone marrow transduced with empty vector (**Supplementary Fig. 13a,b**). Notably, disease nevertheless developed in $CD3\varepsilon\delta\gamma\zeta^{WT}$-$CD3\delta\gamma\varepsilon^{WT}\zeta^M$ mixed bone marrow chimeras, albeit to a lesser extent, which suggested that lymphopenia was not required for disease development (**Supplementary Fig. 13c,d**). Disease in these mice seemed to resolve after about 6 weeks after transfer, which may have been because of the presence of regulatory T cells (T_{reg} cells) derived from wild-type $CD3\varepsilon\delta\gamma\zeta^{WT}$ bone marrow. These data suggest that the autoimmune disease of CD3-mutant mice is exacerbated but not caused by lymphopenia.

T_{reg} cell number and function

We next sought to determine whether autoimmune disease was due to a breakdown of peripheral or central tolerance. T_{reg} cells are a critical subpopulation of $CD4^+$ T cells that are essential for maintaining self tolerance and preventing autoimmunity[21–25] and for limiting chronic inflammatory diseases, such as asthma and inflammatory bowel disease[26,27]. T_{reg} cells are key in enforcing peripheral tolerance, and their absence can cause profound autoimmunity[28,29]. The percentage of splenic $Foxp3^+$ T_{reg} cells was essentially normal in four groups of CD3-mutant mice with a range of wild-type ITAM numbers (**Fig. 4a**). Immunofluorescence analysis suggested that $CD3\delta\gamma\varepsilon^{WT}\zeta^M$ $Foxp3^+$ T_{reg} cells were able to migrate into the lung and liver lesions of autoimmune mice (data not shown). Notably, despite their TCR signaling deficiencies, $CD3\delta\gamma\varepsilon^{WT}\zeta^M$ T_{reg} cells had nearly normal suppressive activity relative to that of their wild-type counterparts in controlling the proliferation of fully functional naive effector T cells *in vitro* (**Fig. 4b**). Thus, the autoimmune disease that developed in CD3-mutant mice seemed to do so in the presence of a normal proportion of functionally competent $Foxp3^+$ T_{reg} cells. However, it is possible that this population had some functional deficiency *in vivo*.

Lower CD3 signaling converts negative to positive selection

It was possible that the autoimmune disease of CD3-mutant mice could have been due to a breakdown in central tolerance[30,31]. For example, self-reactive thymocytes that are normally deleted by negative selection could instead have been positively selected because of lower TCR signaling capacity. To test that hypothesis directly, we expressed wild-type or mutant CD3 molecules together in cells bearing a transgene encoding the MataHari TCR (V_β8.3, specific for the male antigen Uty[32,33]). We transduced bone marrow from male or female CD3εζ-KO *Rag1*$^{-/-}$ mice with MataHari TCRα-2A-TCRβ retrovirus plus either wild-type or mutant CD3 retrovirus and then used that bone marrow to reconstitute irradiated *Rag1*$^{-/-}$ recipients. In $CD3\delta\gamma\varepsilon\zeta^{WT}$ MataHari female mice, a uniform population of $CD8^+V\beta8.3^+$ T cells was present in the periphery (**Fig. 5a–c**). However, in the presence of the male self antigen, T cells were deleted, as indicated by the lack of GFP^+ cells in the blood (**Fig. 5a,b**). Some T cells accumulated in the spleen but had low expression of CD8, lacked V_β8.3 TCR expression and failed to produce IFN-γ after antigen stimulation (**Fig. 5c** and data not shown). Such features are indicative of effective negative selection and peripheral tolerance induction[32,34]. In contrast, male mice expressing three of four mutant

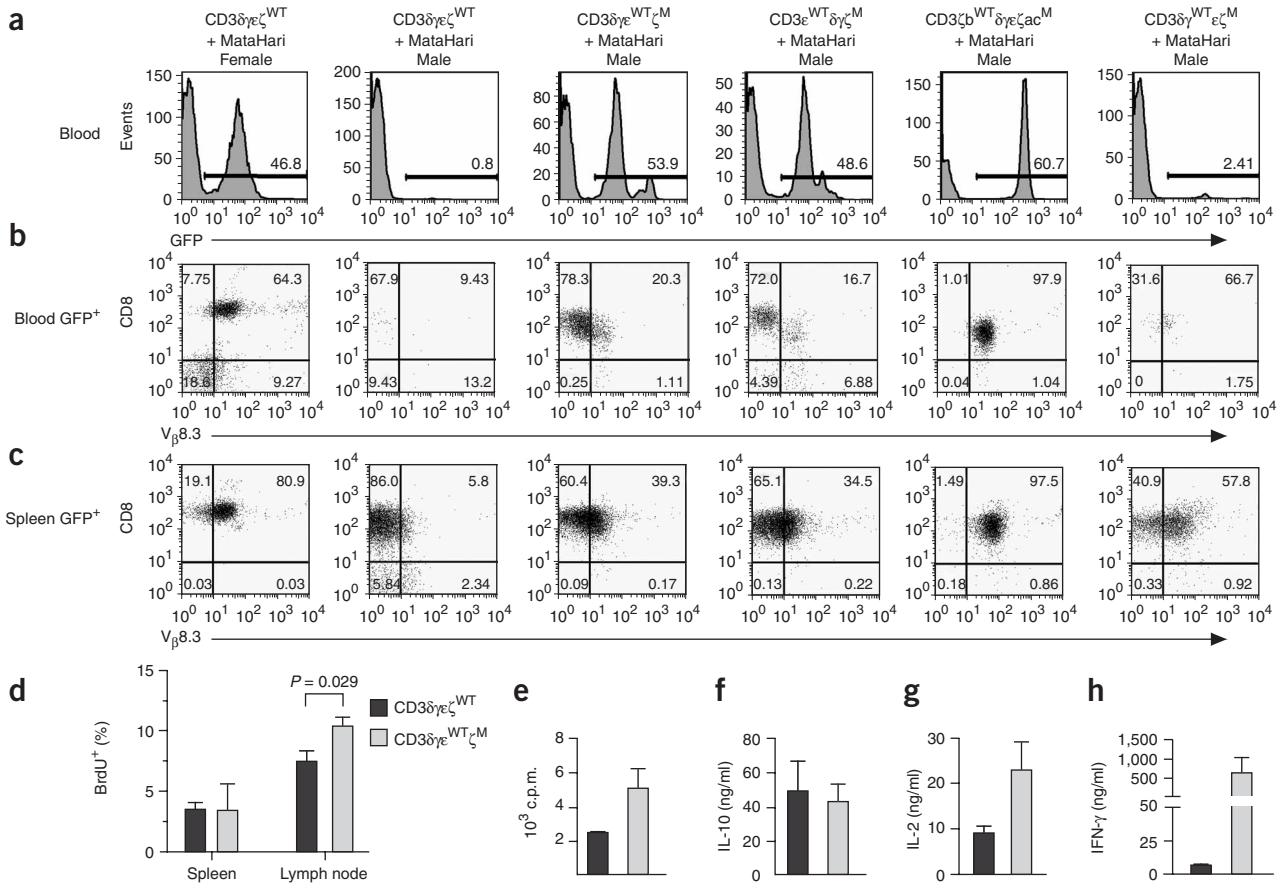


Figure 5 Lower signaling converts negative selection into positive selection and generates IFN-γ-producing autoreactive T cells. (**a**–**c**) Flow cytometry of cells from blood (**a**,**b**) and spleens (**c**) of *Rag1*$^{-/-}$ mice 8 weeks after they were reconstituted with CD3εζ-KO *Rag1*$^{-/-}$ bone marrow transduced with retrovirus encoding MataHari TCRαβ and retrovirus encoding wild-type or mutant versions of CD3δγεζ. Blood (**a**), peripheral blood lymphocytes gated on live lymphocytes (with a forward scatter–side scatter gate); blood GFP$^+$ (**b**), peripheral blood lymphocytes gated on GFP$^+$ live lymphocytes (with FL1 and a forward scatter–side scatter gate). Data are representative of 5–15 mice per group in one to three separate experiments. (**d**) Proliferation of splenocytes and lymph node cells from CD3δγεζ^{WT} and CD3δγε^{WT}ζ^{M} retrogenic mice given drinking water containing BrdU for 8 d, assessed 4 weeks after transplant by staining for CD4 and BrdU and gating on CD4$^+$ lymphocytes. Data are representative of two experiments with four to five mice per group. (**e**–**h**) Proliferation (**e**) and cytokine secretion (**f**–**h**) of cells from the brachial, cervical and mesenteric lymph nodes of CD3δγεζ^{WT} and CD3δγε^{WT}ζ^{M} retrogenic mice, assessed 6 weeks after transplant by being plated for 48 h as single-cell suspensions in 96-well round-bottomed plates. (**e**) Cells pulsed for a further 24 h with [^{3}H]thymidine. (**f**–**h**) Analysis of cytokines in 50 μl of the supernatant immediately after 48 h of culture. Data are the mean and s.e.m. of two to five mice per group in two separate experiments.

CD3 ITAM combinations had a high percentage of GFP$^+$ cells in the blood, and all groups had a substantial percentage of CD8$^+$Vβ8.3$^+$ T cells in the spleen (**Fig. 5a–c**). However, these T cells were unresponsive to stimulation with cognate antigen, consistent with previous observations[35] (data not shown). These data suggest that a lower wild-type ITAM number resulted in positive rather than negative selection of self-reactive T cells.

If there were a failure of negative selection in CD3-mutant mice that developed autoimmunity, autoreactive T cells would be expected in the periphery of sick mice. We first assessed whether T cell proliferation was higher in CD3-mutant mice by *in vivo* labeling with the thymidine analog BrdU (5-bromodeoxyuridine). Whereas the percentage of CD3δγεζ^{WT} and CD3δγε^{WT}ζ^{M} BrdU$^+$CD4$^+$ T cells in the spleen was identical, CD3δγε^{WT}ζ^{M} mice had 43% more BrdU$^+$CD4$^+$ T cells than CD3δγεζ^{WT} mice had in lymph nodes draining the sites of inflammatory lesions (**Fig. 5d**). In addition, CD3δγε^{WT}ζ^{M} T cells showed more *ex vivo* proliferation in response to lymph node–resident, self antigen–expressing antigen-presenting cells (**Fig. 5e**). These findings are notable, given that CD3-mutant T cells had a distinct proliferative defect (**Fig. 3a,b**). Finally, although CD3δγε^{WT}ζ^{M} T cells produced minimal IL-10 and only twofold more IL-2 than CD3δγεζ^{WT} T cells did, they secreted 100-fold more IFN-γ (**Fig. 5f–h**). These data collectively suggest that autoreactive T cells with lower CD3 signaling capacity escape negative selection, show enhanced proliferation in the periphery and produce substantial IFN-γ in response to antigen-presenting cells expressing self antigen.

DISCUSSION

Our data have identified an unexpected difference in the number TCR-CD3 ITAMs required to drive distinct T cell events *in vivo* and have shown that lower wild-type ITAM numbers, which presumably 'translate' into lower TCR signal strength, resulted in a lethal autoimmune disease. Our data are consistent with the following model. Lower TCR signal strength leads to a breakdown of central tolerance and a failure to delete self-reactive T cells in the thymus, which are instead positively selected. It is also possible that the TCRs on these autoreactive T cells have a higher affinity for self peptide–MHC complexes[20]. Such T cells subsequently escape into the

periphery, where their high affinity for self peptide–MHC causes rampant T cell activation. Although the proliferative potential of the CD3-mutant T cells was low, their autoreactive status still induced some proliferation in lymph nodes draining sites of inflammatory lesions. Autoimmunity was transferred only with $CD4^+$ T cells, consistent with their predominance in inflammatory lesions. Those activated T cells produced substantial amounts of IFN-γ, which led to considerable macrophage activation. Such observations are reminiscent of the enhanced IFN-γ production and inflammatory bowel disease noted in T cells from mice lacking both CD3ζη and the receptor FcRγ[36]. Notably, in some cases, our CD3-mutant mice also developed autoantibodies; this may have been more substantial had the mice survived longer. This proinflammatory milieu led to widespread vasculitis, especially in the lungs and liver, enterocolitis and ultimately death.

Peripheral tolerance seemed to be intact, as the proportion and functionality of $Foxp3^+$ T_{reg} cells in CD3-mutant mice was similar to that of their wild-type counterparts. However, we cannot rule out the possibility of a partial defect that resulted in lower suppressive function *in vivo*. It is also possible that T_{reg} cells failed to control disease in these mutant mice because inflammatory cytokines overrode their regulatory ability.

Although low thymic T cell output correlated with the development of autoimmunity, there were distinct differences in the disease of CD3-mutant mice versus lymphopenia-driven inflammation, which made an autoimmune etiology for the former far more probable. Notably, chimeric mice with both CD3-wild-type and CD3-mutant T cells, which were thus not lymphopenic, still became ill, which suggested that lymphopenia was not a prerequisite for disease. However, it is still possible that lymphopenia exacerbated and/or potentiated the severity of this autoimmune disease.

The main reason for a TCR-CD3 complex to have a high ITAM number seems to be quantitative rather than qualitative. A defined number of wild-type ITAMs was required for each T cell developmental and homeostatic parameter, which indicated that a predetermined strength of signal or threshold was needed to initiate pre-TCR signaling, central tolerance, homeostatic expansion and maintenance of the $CD4^+$ cell/$CD8^+$ cell ratio. Notably, effector function seemed to be governed by somewhat different rules, as the lower proliferative potential had a linear relationship with ITAM number, whereas cytokine production was relatively unaffected by lower wild-type CD3 ITAM number. Such findings may indicate a requirement for a regulated, 'rheostat-like' control of proliferation while maintaining the ability to produce substantial amounts of cytokines in the presence of very low ligand densities. The minimum number of ITAMs required for cytokine production may explain the importance of SOCS proteins and their function in preventing cytokine signaling during T cell development[37].

Although many of the CD3 ITAMs seemed interchangeable, specific ITAMs had a secondary qualitative influence on the development and function of T cells. For example, there were differences between the CD3δ and CD3γ ITAMs, which differ in only four amino acid residues. The most notable example of a qualitative influence of ITAMs was in the four groups with six functional ITAMs; among these, two groups developed profound autoimmunity, whereas the other two showed no disease manifestations. Whether this apparent modulatory function of individual ITAMs was due to sequence differences and/or their location in the complex remains to be determined.

Several studies have shown that impaired TCR signaling can lead to autoimmunity in both mice and humans[38–40]. For example, T cells from patients with systemic lupus erythematosus have antigen receptor–mediated signaling aberrations[39]. Indeed, truncations of the CD3ζ cytoplasmic tail, which lead to lower TCR surface expression and signaling, have been detected in humans with autoimmunity[41,42]. Furthermore, other TCR-CD3–mutant mice develop chronic inflammation, albeit with slower kinetics than those of our CD3-mutant mice[15,36,43]. Thus, from an evolutionary perspective, it may be important to have an excess number of ITAMs, and thus scalable signaling, in the TCR-CD3 complex to ensure effective negative selection and some protection from deficiencies in another part of the signaling cascade. Its broad dynamic range allows the TCR to mediate diverse actions, such as positive versus negative signaling and proliferation versus cytokine production, with essentially the same signaling machinery.

METHODS

Mice. *Rag1*$^{-/-}$, CD3ζ-KO and C57BL/6J mice were from Jackson Laboratories. CD3εζ-KO mice, generated by the crossing of *Cd3e*$^{\Delta P/\Delta P}$ mice (provided by C. Terhorst) with CD3ζ-KO mice, have been described[9,10]. CD3ζ-KO Tg1-6^M mice were from N. van Oers[44]. Mice transgenic for the 3A9 TCR were from M. Davis[45]; this HEL (48–62)-specific TCR is H-$2A^k$ restricted but will mediate T cell development on the H-2^b background of the mice used in these experiments. *Rag1*$^{-/-}$, CD3εζ-KO, CD3εζ-KO *Rag1*$^{-/-}$, CD3ζ-KO, 3A9.CD3ζ-KO and CD3ζ-KO Tg1-6^M mice were re-derived, bred and maintained as helicobacter-free, citrobacter-free, specific pathogen–free colonies at St. Jude. All animal experiments were done according to national, state and institutional guidelines in a helicobacter-free and specific pathogen–free facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude.

Generation of CD3 multicistronic vectors. Constructs linked to the 2A peptide were generated by recombinant PCR and were cloned into pMIG, a mouse stem cell virus–based retroviral vector containing an internal ribosomal entry site–GFP cassette, as described[9,46,47]. Additional restriction sites were introduced, and an additional construct was designed with substitution of each tyrosine with phenylalanine in all CD3-chain ITAMs. Subsequent CD3-mutant constructs were then generated by the appropriate subcloning.

Retrovirus-producing cells. Cell lines producing retrovirus were generated as described[48–50] with some modifications[9]. Human embryonic kidney 293T cells were transiently transfected with 4 μg pMIG vector with or without sequences encoding TCRαβ, together with packaging and envelope vectors, with the TransIt 293T transfection reagent (Mirus). GP+E86 cells, a mouse embryonic fibroblast 3T3–based packaging cell line, were transduced every 12 h for 3–4 d with virus and polybrene (6 μg/ml) until a viral titer above 1×10^5 viruses per ml after 24 h was obtained.

Retrovirus-mediated stem cell gene transfer. Retroviral transduction of mouse bone marrow cells was done as described[9,11]. Bone marrow was collected from 8- to 10-week-old donor mice 48 h after treatment with 150 mg 5-fluoruracil (Pharmacia & UpJohn) per kg body weight. Bone marrow cells were cultured for 48 h in complete DMEM with 20% (vol/vol) FBS, mouse IL-3 (20 ng/ml), human IL-6 (50 ng/ml) and mouse stem cell factor (50 ng/ml; Biosource-Invitrogen). Cells were then cultured together for a further 48 h with irradiated (1,200 rads) retrovirus-producing cell lines plus polybrene (6 μg/ml) and cytokines as described above. Nonadherent transduced bone marrow cells were collected and washed and were resuspended in PBS containing 2% (vol/vol) FBS plus heparin (20 U/ml). Bone marrow cells (4×10^6 cells per mouse) were injected through the tail vein into irradiated (450 rads) *Rag1*$^{-/-}$ recipient mice. After initial mice were noted to become sick and die within 5–7 weeks of transfer, all further experiments were completed when the mice were first noted to be sick (lethargy, hunched posture, ruffled coat, dehydration and squinting). Necropsy showed that the few sick wild-type mice had no inflammation and were sick because of an unrelated cause.

Additional methods. Information on histology and immunofluorescence, flow cytometry and cell sorting, proliferation and cytokine measurements, T_{reg} cell assays, autoantigen array production and probing, and microarray data analysis is available in the **Supplementary Methods** online.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A000552, A000550, A000549 and A000553.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

All authors contributed to discussions of experimental design, data analysis and/or editing of the manuscript; J.H. did all experiments unless stated otherwise; H.W., K.D.E. and C.J.W. generated mice for the immunofluorescence microscopy and autoantibody analysis, did the T_{reg} cell experiments, chimera-lymphopenia experiments, some of the flow cytometry analysis and the BrdU and lymph node assays; K.L.B. did all the histological analysis and scoring while 'blinded' to sample identity; K.F. assisted in cellular isolation and analysis and managed the breeding colonies; Z.B. and R.S. did the immunofluorescence microscopy, H.S., A.C. and P.J.U. did the autoantibody analysis; N.S.C.v.O. provided the CD3ζ-KO Tg1-6^{M}-transgenic mice and discussions about their use; J.H. and D.A.A.V. wrote the manuscript; and D.A.A.V. conceptualized and directed the project.

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