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Opposing functions of *Zap70* for immunity and tolerance titrate differentially in response to inherited single nucleotide substitutions

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Summary

Null mutations that cripple TCR antigen recognition explain rare primary immunodeficiencies, but it is not understood why more common single nucleotide substitutions that create subtle TCR signaling defects are paradoxically associated with autoimmunity in some instances but not others. Here we analyse how a set of *Zap70* gene variants, which alter individual catalytic cleft residues to produce stepwise decreases in TCR signaling, impact upon opposing TCR functions in different T cells and upon regulation of the immune system as a whole. One *Zap70* variant, *murdock*, that moderately decreases TCR signaling and thymic selection is tolerated at the system level, while a more severe *Zap70* defect, *mrtless*, abolishes thymic positive selection and Causes immunodeficiency. At a threshold between these two states, negative selection and Foxp3⁺ regulatory T cell formation are compromised to a greater degree than positive selection and memory/effector cell formation. This cellular imbalance can no longer be controlled at the system level, resulting in formation of autoantibodies and hyper-IgE. The pleiotropic functions of *Zap70* and their differential response to graded genetic variation provide a paradigm for understanding common genetic variation in humans.

Introduction

Precisely how genetic variation affects our susceptibility to infection, autoimmunity and allergy is only scarcely understood. The largest sources of human genetic variation are

Competing Interests Statement

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

Antigen recognition and T cell activation by the T cell receptor (TCR) is one of the most well defined pathways of the immune system, having been dissected in mice and humans with loss of function alleles in many of the critical components (Kane et al., 2000). Loss of key enzymes in this pathway, such as ZAP-70 tyrosine kinase, results in severe immunodeficiency in man and mouse due to failure of the TCR to signal T cell maturation in the thymus and T cell activation in the periphery (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994; Negishi et al., 1995; Wiest et al., 1997). By contrast, single nucleotide substitutions in Zap70 and other molecules in this pathway have recently been identified that do not abolish T cell differentiation or activation but are associated, paradoxically, with autoimmunity and immunopathology. In mice, an amino acid substitution in the C-terminal SH2 domain of ZAP-70 (W163C: Zap70^{skg}) that decreases TCR signaling also causes autoimmune arthritis when combined with subclinical fungal infection and other genetic factors (Sakaguchi et al., 2003; Yoshitomi et al., 2005). Similarly, a single amino acid substitution in the protein Linker of Activated T cells, altering a tyrosine normally phosphorylated by ZAP-70 (LAT^{Y136F}), diminishes TCR signaling yet causes a severe inflammatory and hyper-IgE syndrome in mice (Aguado et al., 2002; Sommers et al., 2002). In man, a common nucleotide and amino acid substitution in a protein tyrosine phosphatase, PTPN22^{R620W}, acts upon LCK upstream of ZAP-70 to decrease TCR signaling (Vang et al., 2005), and has been widely associated with rheumatoid arthritis, Type 1 diabetes, systemic lupus erythematosus and autoimmune thyroid disease (Begovich et al., 2004; Bottini et al., 2004; Criswell et al., 2005; Kyogoku et al., 2004; Velaga et al., 2004). Also upstream of ZAP-70 is a promoter polymorphism that diminishes expression of SH2D2A, encoding T Cell Specific Adaptor (TSAd) required for optimal activation of ZAP-70 by LCK (Marti et al., 2006). This polymorphism is associated with multiple sclerosis and juvenile rheumatoid arthritis (Dai et al., 2001; Smerdel et al., 2004). Many more examples of partial defects in the TCR pathway will no doubt emerge as new tools to resequence genes or detect copy number variation are applied. Using this genetic information will hinge upon understanding how partial defects in the efficiency of TCR signaling impact upon regulation of autoimmunity and immunopathology.

It is not known how inherited defects in TCR signaling translate into outcomes such as autoimmunity, hyper-IgE and immunopathology in some instances but not in others. One hypothesis is that the paradoxical variants create a qualitative biochemical imbalance among parallel signaling pathways within individual T cells, for instance crippling inhibitory feedback events to a greater extent than activating events, such that T cell hyper-responsiveness or dysregulation occurs. For example, the LAT^{Y136F} point mutation selectively diminishes docking and activation of phospholipase C gamma while preserving docking sites for many other signaling molecules in the LAT scaffold, diminishing calcium but not ERK signaling. Similarly, the amino acid substitution in the SH2 domain of ZAP-70^{skg} diminishes ZAP-70 binding to phosphorylated TCR zeta chain ITAMs, potentially increasing the recruitment and activation of parallel signaling molecules. An alternative hypothesis is that simple quantitative differences in TCR signaling in some cases create a qualitative cellular imbalance, due to opposing actions of the TCR in different T cells. In the thymus, TCR signaling triggers divergent functions of positive and negative selection, depending upon the strength of self peptide/MHC binding to unique TCRs on each

thymocyte (Daniels et al., 2006). Diminished TCR signaling caused by the ZAP-70^{skg} or LAT^{Y136F} alleles causes thymocytes with strongly self-reactive TCRs to H-Y antigen to be positively selected instead of negatively selected (Sakaguchi et al., 2003; Sommers et al., 2005). This quantitative change in the positive versus negative selection threshold might not be expected to result in autoimmunity, however, since it also changes the activation threshold for peripheral T cells such that strongly self-reactive T cells that are positively selected cannot be activated by these antigens in the periphery (Sommers et al., 2005). TCR signaling is also needed for the formation and activity of CD4⁺Foxp3⁺ regulatory T cells, and a recent study suggests that deficiency of this inhibitory subset accounts for the immunopathology and hyper-IgE syndrome in LAT^{Y136F} mice (Koonpaew et al., 2006). TCR signaling is also needed to maintain peripheral naïve T cells, and when populations of these cells are reduced, availability of IL-7 increases to facilitate activation of self-reactive T cells (Baccala and Theofilopoulos, 2005).

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

Results

Identification of the Zap70 variant strain murdock

The starting point for this study was a mouse genome-wide screen for N-ethyl-Nnitrosourea-induced single nucleotide substitutions affecting immune regulation (Nelms and Goodnow, 2001). We identified one variant C57BL/6 strain, murdock (mrd), with fewer peripheral T cells of the naïve CD44^{lo} subset (Figure 1A). This T cell trait was inherited in a recessive Mendelian fashion on the C57BL/6 background and in an F2 intercross with the NOD. $H2^k$ strain (Figure 1B) where 39 of 156 individuals (25%) exhibited the *murdock* phenotype. A genome-wide scan of pooled DNA from affected F2 mice linked the trait to D1Mit212 on chromosome 1, with further typing localizing the mutation to an ~11Mb interval between D1Mit410 and D1Mit245 (Figure 1C). Within this interval the Zap70 gene (37.06 Mb) was a prime candidate, considering the arrest of $\alpha\beta$ T cell development in Zap70-deficient mice (Negishi et al., 1995). Sequencing of Zap70 cDNA from mrd/mrd homozygotes revealed a single A to T transversion at base 1207 that was not present in the parental C57BL/6 stock (Figure 1D). The mutation substitutes an isoleucine codon for phenylalanine (I367F) within the catalytic kinase domain (Figure 1D). This would be predicted to alter the dimensions of the ATP-binding pocket within the catalytic cleft. For comparison, the W504R mrtless mutation (described below) changes a conserved residue in the activation loop within the catalytic site. The murdock I366F mutation caused no measurable difference in ZAP-70 protein expression between mrd/mrd and +/+ C57BL/6 thymocytes (Figure 1E). In a co-transfection kinase assay, however, the corresponding I368F substitution in human ZAP-70 markedly decreased total (α P-Tyr) and substratespecific (aNTAL) phosphorylation (Figure 1F).

To confirm that the $Zap70^{mrd}$ I367F substitution was responsible for the *murdock* T cell phenotype we performed a genetic complementation cross with a second mouse strain

bearing a mutant Zap70 gene, mrtless (mrt), discovered by the same screen in a parallel pedigree (Yates et al., in preparation). The Zap70^{mrt} allele results from a W504R codon change within the catalytic site, which in the homozygous state reduces ZAP-70 protein to 25% of wild-type levels and almost completely arrests T cell development at the CD4⁺CD8⁺ thymocyte stage. From a Zap70^{mrd/mrd} by Zap70^{mrt/+} cross, approximately half the offspring exhibited reduced percentages of CD4⁺ peripheral T cells (Figure 1G), confirming that the murdock and mrtless T cell traits result from non-complementary alleles.

Spontaneous production of autoantibodies and excessive secretion of IgE and IgG1 in Zap70^{mrd/mrt} mice

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

This unique increase in spontaneous antibody secretion in $Zap70^{mrd/mrt}$ mice contrasted with diminished acute IgG antibody response to immunization (Figure 2C). Primary T_H1 responses to heat-killed *Bordetella pertussis* were particularly sensitive to Zap70 defects, being equally depressed to ~2% of the wild-type response in each of the three Zap70 mutant strains. Primary and secondary responses to chicken gammaglobulin (CGG) and secondary responses to Arsonate hapten (ABA) conjugated to CGG were absent in $Zap70^{mrt/mrt}$ mice (as might be expected given severe T cell deficiency in these animals) and tended to be lower in $Zap70^{mrd/mrt}$ compound heterozygotes than in $Zap70^{mrd/mrd}$ animals. Antibody responses to the T-independent antigen NP-Ficoll were normal across all Zap70 mutant strains.

Graded and threshold decreases in helper T cell subsets

To understand why autoantibodies, hypergammaglobulinemia and hyper-IgE were selectively triggered in animals co-expressing two different ZAP-70 catalytic site mutants, we first compared the impact of the different Zap70 genetic states on the number of peripheral CD4 T cells (Figure 3). As expected, Zap70^{mrt} homozygous mice had very few CD4 cells. By contrast, mice homozygous for Zap70^{mrd} had half the number of peripheral CD4 cells of wild-type controls (Figure 3A,B) - this being due solely to a 70% decrease in naïve (CD44^{lo}) CD4 cells while activated/memory (CD44^{hi}) CD4⁺ cells remained in normal numbers (Figure 3D). The number of CD4410 CD4 cells was decreased by a further 60% in compound heterozygous Zap70^{mrd/mrt} animals, while there was still no impact on the number of CD44^{hi} cells. Thus, Zap70 genotype had a graded impact on naïve CD4 T cells in the order *mrd/mrd>mrd/mrt>mrt/mrt*, whereas a distinct threshold was required before the memory/effector compartment was numerically affected. Graded effects on naïve CD4 cell number were mirrored in TCR-induced calcium responses, which were subtly decreased in T cells homozygous for Zap70^{mrd/mrd} but dramatically decreased in Zap70^{mrd/mrt} T cells (Figure 3E). A distinct threshold also applied to the CD8 T cell subset, which was not significantly reduced in Zap70^{mrd/mrd} or Zap70^{mrd/mrt} individuals (due to a contraction in CD44^{lo} cells and expansion of the CD44^{hi} subset) but was greatly decreased in Zap70^{mrt/mrt} animals. The preferential effect of the partial Zap70 defects on naïve CD4 cells caused a progressively skewed CD8:CD4 ratio that was most extreme in the Zap70^{mrd/mrt} combination (Figure 3C). These different thresholds for effects on CD44^{lo} and CD44^{hi} subsets of CD4 and CD8 cells are consistent with the evidence that these subsets vary in

their relative dependence on TCR signals for persistence (Boyman et al., 2007;Caserta and Zamoyska, 2007).

To understand the origins of graded decreases in naïve CD4 cells we examined the impact of the different Zap70 states upon thymocyte differentiation (Figure 4). Formation of $CD4^{-}CD8^{+}$ thymocytes was not measurably affected by the mild Zap70^{mrd} allele, only slightly decreased by the Zap70^{mrd/mrt} combination, but almost completely abolished by homozygosity for Zap70^{mrt}. By contrast, five different manifestations of TCR signal strength (Azzam et al., 1998;Naramura et al., 2002;Sosinowski et al., 2001) showed stepwise decreases in the order +/+ > mrd/mrd > mrd/mrt > mrt/mrt: CD4⁺CD8⁻ cell numbers (Figure 4A,B); and CD5, CD69, TCR β and CD3 ϵ expression upon DP cells (Figure 4C and Supplementary Figure 1B, C). Incidentally, by using the same parameters, comparison of Zap70^{mrt/mrt} and homozygous null Zap70^{-/-} animals established that Zap70^{mrt/mrt} thymocytes have greater responsiveness to TCR stimulation than thymocytes with no ZAP-70 (Yates et al., in preparation). Decreased TCR and CD3 surface expression also occurred on single positive thymocytes and peripheral T cells in a graded manner with mrd/mrt lower than mrd/mrd (Supp Figure 1C). Thus, inheritance of the mild Zap70^{mrd} allele together with the severe Zap70^{mrt} allele diminishes TCR signaling to an intermediate point between the parental homozygotes (and intermediate between wild-type and null) with the resulting allelic series representing four distinct levels of TCR signaling efficiency.

Graded and threshold defects in thymic tolerance in Zap70^{mrd/mrt} mice

To understand why stepwise decreases in TCR signaling and CD4 T cell formation selectively triggered autoantibodies, hypergammaglobulinemia and hyper-IgE at only one point in our allelic series, we next asked whether there was a threshold reduction in TCR signaling required to disrupt self tolerance by clonal deletion. Since TCR signaling thresholds for negative selection are likely to differ for different TCRs, we sought a consensus by analysing polyclonal T cell populations expressing V β 5 or V β 11 TCRs with a range of affinities for MMTV self-superantigen complexed with I-E or I-A MHC molecules (Fink et al., 1994; Kappler et al., 1987). In mice with wild-type Zap70, a prominent subset of peripheral CD4 and CD8 T cells express V β 5 or V β 11 in I-E-deficient $H2^b$ animals that only express I-A, an MHC molecule that presents the self-superantigen inefficiently (Figure 5A, open circles). These V β 5 or V β 11 cells are eliminated by thymic negative selection in $H2^k$ animals expressing I-E, which efficiently presents self-superantigen to the T cells (Figure 5A, filled circles). Homozygosity for the mild Zap70^{mrd} defect interfered with thymic negative selection in I-E⁺ $H2^k$ animals, completely precluding negative selection of VB11 CD8 cells (the least efficiently deleted subset in wild-type mice) and allowing a substantial subset of V β 11 CD4 and V β 5 CD8 T cells to escape negative selection (Figure 5A). These tolerance events exhibited a low threshold for disruption, since they were not further exaggerated in Zap70^{mrd/mrt} compound heterozygotes with a more severe TCR signaling defect. By contrast, clonal deletion of VB5 CD4 T cells by I-E/MMTV exhibited a higher threshold before disruption, since it was mostly intact in Zap70^{mrd/mrd} mice and only became defective in Zap70^{mrd/mrt} animals. Peripheral deletion of V_{β5} CD4 T cells by I-A/ MMTV in $H2^b$ animals (Fink et al., 1994) showed a graded deficit in response to different TCR signaling lesions, with twice as many cells remaining in Zap70^{mrd/mrd} animals and three times as many in Zap70^{mrd/mrt} animals.

To analyze the impact on positive and negative selection of CD4 cells bearing a single TCR specificity, *Zap70^{mrd}* and *Zap70^{mrt}* alleles were crossed with transgenic mice expressing a hen egg lysozyme (HEL)/I-A^k specific TCR (3A9) and insHEL expressed in pancreatic islet beta cells and thymic medullary epithelium (Akkaraju et al., 1997; Liston et al., 2003). In mice lacking insHEL, the number of CD4 single positive cells expressing the 3A9 TCR was diminished sixty-fold in the thymus (Figure 5B,D, and E) and ten-fold in the spleen (Figure

5C) of animals homozygous for the mild $Zap70^{mrd}$ allele. The small number of clonotypepositive CD4 cells that are positively selected in spite of the mrd mutation may do so either based on signals through the 3A9 receptor, or through a co-expressed endogenous receptor. In either case, these data demonstrate that positive selection of some TCRs is acutely sensitive to modest changes in TCR signal strength, thus extending the evidence in Figure 4A that half as many $CD4^+CD8^-$ thymocytes matured in Zap70^{mrd/mrd} thymi. Essentially no clonotype-positive CD4 cells were present in TCR-transgenic Zap70^{mrd/mrt} compound heterozygotes (not depicted). In the thymus of two sets of TCR insHEL double transgenic mice analysed there was little decrease in the number of clonotype-bearing CD4 cells in Zap70^{mrd/mrd} double transgenics compared to Zap70^{mrd/mrd} TCR animals, and their absolute number was higher than the corresponding wild-type double transgenic siblings analysed at the same time (Figures 5D, E). The persistence of clonotype-bright CD4 SP thymocytes in Zap70^{mrd/mrd} TCR insHEL animals could either be a direct effect of decreased TCR signalling, an indirect effect of failure to exclude an endogenous TCR alpha chain, or a combination of the two. Regardless, these data reinforce the evidence in Figure 5a that negative selection of some TCRs has a low threshold for disruption by inherited changes in TCR signal strength.

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

Immune dysregulation caused by Zap70^{mrd/mrt} T cells is not suppressed by +/+ T cells

The more severe defects in CD4 cell clonal deletion and in CD4⁺Foxp3⁺ regulatory T cells in Zap70^{mrd/mrt} compound heterozygous mice provided two alternative, although nonexclusive, explanations for the selective occurrence of autoantibodies, hypergammaglobulinemia and hyper-IgE. To test these alternatives, we performed *in vivo* cell mixing experiments to ask whether or not dysregulated help for antibody secretion was a cell autonomous trait of Zap70^{mrd/mrt} T cells or if it could be suppressed in the presence of wild-type T cells. First, we injected large numbers ($\sim 3 \times 10^7$) of wild-type spleen cells into young Zap70^{mrd/mrt} mice. Although this did not correct autoantibody or hyper-IgE secretion in Zap70^{mrd/mrt} animals (not depicted), the same procedure corrected a similar antibody dysregulation in a SLP-76 hypomorphic *lcp2* mutant mouse strain housed under identical conditions (Siggs et al, in preparation). Second, we reconstituted the hematopoietic system of irradiated RAG1-deficient mice with mixtures of fetal liver from CD45.1 Zap70^{mrd/mrt} and wild-type or Foxp3-deficient CD45.2 donors. All recipients of 100% Foxp3-deficient fetal liver, but not mixtures of Foxp3-deficient and Zap70^{mrd/mrt} liver, became ill 6-7 weeks post-reconstitution, indicating a functional competence of Zap70^{mrd/mrt} regulatory T cells sufficient to suppress *scurfy* syndrome. In vivo Zap70^{mrd/mrt} Treg activity was also apparent

when Foxp3-deficient splenocytes were transferred into RAG1-deficient or Zap70^{mrd/mrt} recipients, because RAG1-deficient but not Zap70^{mrd/mrt} recipients succumbed to a progressive loss of weight characteristic of inflammatory bowel disease (supplementary Figure 2). With respect to serology, a high background of serum autoantibodies in all reconstituted RAG recipients, regardless of donor cell type, precluded any analysis of the Zap70^{mrd/mrt} contribution to this trait. By contrast, hyper-IgE occurred in all Zap70^{mrd/mrt} reconstituted animals and none of the control animals reconstituted with wild type fetal liver (Figure 7A). In the mixed chimeras with wild-type or *Foxp3*-deficient CD45.1 donors, there was no significant decrease in IgE compared to the groups that received only Zap70^{mrd/mrt} fetal liver (Figure 7A). Flow cytometric analysis of the mixed chimeras nevertheless demonstrated that between 10% and 80% of the peripheral CD4 cells in these animals were derived from the Zap70 wildtype CD45.1⁺ donor (Figure 7B). Moreover, the mixed chimeras displayed high IgE in spite of the presence of a high proportion of wild-type CD4⁺Foxp3⁺ T cells (Figure 7C). Induction of IgE secretion is therefore a cell autonomous trait of Zap70^{mrd/mrt} T cells that is most likely due to the increased frequency of self-reactive CD4 T cells, and cannot be complemented by wild-type CD4⁺Foxp3⁺ cells.

Discussion

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

Autoantibodies, hypergammaglobulinemia and elevated IgE occur in a number of mouse models where point mutations partially compromise components of the TCR signaling pathway, yet are absent in others. In simplest terms, our analysis of graded defects in ZAP-70 explain these paradoxical outcomes as resulting from lower robustness of regulatory mechanisms compared to helper mechanisms in CD4 cells, such that dysregulated help manifests when inherited defects reduce TCR signaling to a critical intermediate point. Considering our *Zap70* series together with other models, the pathogenesis of immune

dysregulation in individuals with diminished TCR signaling appears multifactorial and pleiotropic, with variable contributions from defects in thymic deletion, regulatory T cell formation and function, lymphopenia, and immune deficiency/opportunistic infection.

Failure of thymic deletion and autologous responses of CD4 $T_H 2$ or $T_H 17$ cells has been suggested to cause antibody dysregulation in LAT^{Y136F} mice (Aguado et al., 2002; Sommers et al., 2005) and arthritis and hypergammaglobulinemia in *Zap70^{skg}* mice (Hirota et al., 2007; Sakaguchi and Sakaguchi, 2005). Nevertheless, this tolerance defect is insufficient to cause dysregulation in *Zap70^{skg}* mice maintained in an SPF facility or on a different inbred background (Jiang et al., 2005). Similarly, there is already a marked failure of thymic deletion in *Zap70^{mrd/mrd}* mice that does not translate into autoantibodies or hypergammaglobulinemia, whereas a more severe failure of thymic deletion in *Zap70^{mrd/mrd}* individuals is accompanied by antibody dysregulation. Intuitively, one might expect that a decrease in TCR signaling for thymic deletion would be cancelled out by an equal decrease in TCR signaling for activation in the periphery, such that the system tolerates varying efficiencies of thymic deletion. The fact that hyper-IgE in *Zap70^{mrd/mrt}* mice is dominant in mixed chimeras, and is not suppressed by equal numbers of normal T cells or regulatory T cells, indicates a T cell autonomous defect such as escape from thymic deletion.

The role of lymphopenia and regulatory T cell deficiency in immune dysregulation from partial TCR signaling defects also appears to contribute to varying degrees. Elevated IgE is characteristic of regulatory T cell deficiency caused by mouse and human FOXP3 mutations, and of hypomorphic mouse and human RAG mutations where there is a limited repertoire of competing T cells (Khiong et al., 2007; Marrella et al., 2007; Villa et al., 1998). While antibody dysregulation in our Zap70 allelic series correlates with a marked drop in thymic Foxp3⁺ CD4 cells and decreased CD25 expression on the remaining cells, hyper-IgE in Zap70^{mrd/mrt} mice was nevertheless not suppressed by normal T cells or regulatory T cells. This is a point of contrast with LAT^{Y136F} mice, in which pathology can be prevented by the transfer of $2-3 \times 10^5$ CD4⁺CD25⁺ cells (Koonpaew et al., 2006), and with a hypomorphic SLP-76 variant of *lcp2*, in which only 2% CD4⁺ chimerism is sufficient to suppress hyper-IgE (Siggs et al., in preparation). CD4⁺CD25⁺ adoptive transfer has also been reported to prevent arthritis in Zap70^{skg} mice (Hirota et al., 2007; Sakaguchi and Sakaguchi, 2005). These differing outcomes may be explained by the robustness of different T cell mechanisms: autoreactive helper cells in Zap70^{mrd/mrt} mice may retain sufficient TCR signaling for dysregulated helper function in the face of normal regulatory T cells. Alleles that reduce TCR signaling further -Zap70^{skg}, LAT^{Y136F} or a SLP-76 hypomorph - may weaken autoreactive T helper function to an extent that dysregulated help only occurs when regulatory T cells are also crippled.

In addition to intrinsic T cell dysregulation, it has to be considered that defective effector T cell functions that allow opportunistic infection may play a contributory role. Primary immunodeficiency disorders in man are often associated with apparent autoimmune phenomena that stem from a failure to control viral, bacterial and fungal infections (Arkwright et al., 2002). Opportunistic, subclinical fungal infection with *Pneumocystis* or *Aspergillus* has been shown to be an essential co-factor in arthritis development in *Zap70^{skg}* mice, and appears to act by activating dendritic cells, decreasing T cell IL-4 production and increasing T cell interferon gamma production and IgG secretion (Kobayashi et al., 2006; Yoshitomi et al., 2005). These events only transpire in *Zap70^{skg}* mice on a susceptible BALB/c background (Jiang et al., 2005). The finding that dysregulated IgE production in *Zap70^{mrd/mrt}* mice is not suppressed by large numbers of normal T cell in adoptive transfer or mixed chimeras would argue against opportunistic infection, since wild-type T cells should restore immune competence. Moreover, T_H1 responses to *Bordetella* immunization are equally crippled by the *Zap70^{mrd/mrd}* and *Zap70^{mrd/mrt}* alleles, yet only the latter is

associated with dysregulated IgG and IgE secretion. Like the role for regulatory T cell deficiency, there may be variable interplay between primary T cell dysregulation and opportunistic infection in mice with partial TCR signaling lesions, depending upon the extent to which the signaling pathway is compromised and upon other background genes.

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

Experimental Procedures

Mice

murdock (MGI:3614796) was derived from a C57BL/6 G0 male treated intraperitoneally with 100mg/kg *N*-ethyl-*N*-nitrosourea (Sigma) at three weekly intervals. Mutation mapping and DNA sequencing were performed as previously described (Miosge et al., 2002). *murdock* and *mrtless* (MGI:3614790) (Yates et al., in preparation) strains were created on a C57BL/6 background, and analysed on a mixed background with B10.Br (B10.BR-H2k/SgSnJ). 3A9 TCR transgenic (Ho et al., 1994) and ILK-3 insHEL transgenic (Akkaraju et al., 1997) mice were produced on the C57BL/6J background, backcrossed more than seven generations to B10.Br, backcrossed to *murdock* and *mrtless* strains and finally intercrossed. *RAG1*-deficient, *Foxp3*-deficient, NOD.*H2^k* and C57BL/6.Ly5a congenic mice have all been described previously (Fontenot et al., 2003; Lesage et al., 2002; Miosge et al., 2002; Mombaerts et al., 1992). Mice were fixed for *H2^k* and used at 6–18 weeks of age unless otherwise specified. All mice were housed in specific pathogen-free conditions at the Australian Phenomics Facility and Australian Cancer Research Facility (JCSMR), with all animal procedures approved by the Australian National University Animal Ethics and Experimentation Committee.

Flow cytometry

Lymphoid organ suspensions were labelled with FITC-conjugated anti-CD4, anti-CD44, anti-CD45.2 (Ly5b), anti-CD69, anti-TCR β , anti-CD3 ϵ ; PerCP-conjugated anti-CD8 α ; biotinylated anti-V β 5 and anti-V β 11; APC-conjugated streptavidin (BD Pharmingen); PE-conjugated anti-CD25 and anti-CD5 (Caltag); and APC-conjugated anti-CD45.1 (Ly5a) (eBioscience). Intracellular calcium following stimulation with anti-CD3 ϵ (500A2) was measured using a mixture of Ly5-marked splenocytes labelled with Indo-1 (Molecular Probes). anti-Foxp3-FITC intracellular staining was performed according to the manufacturer's recommended protocol (eBioscience).

293T kinase assay

The *murdock* mutation (ZAP-70^{I368F}) was introduced into a human ZAP-70-pcDNA3 construct using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA)

according to the manufacturer's instructions. All other ZAP-70 constructs as well as cDNAs coding for Lck and NTAL have been described previously (Brdicka et al., 2005). NTAL and the endogenous ZAP-70 substrate LAT have subsequently been found to be fully interchangeable in this assay. 293T cells were transfected using Lipofectamine and PLUS Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After 20 hours cells were harvested, washed in PBS and lysed in 60μ L 2× concentrated SDS-PAGE sample buffer. Lysates were ultracentrifuged for 30 minutes at $300,000\times$ g at 20°C and supernatants subjected to SDS-PAGE and immunoblot with antibodies against phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA), NTAL (NAP-4), ZAP-70 (2F3.2) or Lck (1F6, from J. Bolen, Millennium Pharmaceuticals, Cambridge, MA).

Immunizations and ELISA

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

Autoantibodies

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

Fetal liver chimeras

E16 embryonic liver suspensions were prepared in RPMI supplemented with 10% fetal calf serum and mixed in the appropriate ratios. 2×10^5 viable cells were injected intravenously into irradiated *RAG1*-deficient recipients, which were sacrificed for analysis 6–7 weeks later.

Statistical Analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fewer naïve T cells in mice with a ZAP-70 I367F catalytic site substitution, $Zap70^{murdock}$

(A) Representative flow cytometry profiles of CD4 and CD8 expression upon peripheral blood lymphocytes, with representative histograms of CD44 expression on CD4⁺ and CD8⁺ cells from unaffected and *mrd/mrd* mice. (B) Initial generations of the *murdock* pedigree, including mapping intercross, showing affected mice (filled), unaffected animals (unfilled) and untyped (crossed) mice. (C) Meiotic mapping of the *murdock* mutation on chromosome 1. Haplotypes of affected *mrd/mrd* F2IC mice shown in columns: black squares indicate C57BL/6 homozygosity, grey squares indicate C57BL/6-NOD^k heterozygosity. NM, markers not positioned on the current mouse genome assembly. (D) An A to T transversion at *murdock Zap70* cDNA nucleotide 1207 changes codon 367 (human codon 368) from isoleucine to phenylalanine within the ZAP-70 kinase domain. The location of the substituted residue is shown within the human ZAP-70 active kinase domain structure

(PDB: 1U59, complexed with staurosporine), as well as the *mrtless* substitution and key elements of the active site. (E) Anti-ZAP-70 Western blot in C57BL/6 and *mrd/mrd* thymocytes. (F) ZAP-70 kinase assay in 293T cells cotransfected with NTAL, LCK and wild-type (WT) or mutant human *ZAP70* constructs (K369A, kinase-inactive; I368F, *murdock* mutation; vector, no *ZAP70* transfected). After 20 hrs cells were lysed and whole cell lysates immunoblotted with antibodies indicated on right. Net intensity of P-Tyr (×100), as normalized to NTAL in the corresponding sample, is indicated below respective lanes. (G) Pedigree schematic and low number of T cells in compound heterozygous offspring from parents bearing *Zap70 murdock* and *mrtless* missense mutations. Filled symbols denote animals with low T cell numbers.

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Figure 2. Autoantibodies, hypergammaglobulinemia and hyper-IgE in $Zap70^{mrd/mrt}$, but not $Zap70^{mrd/mrd}$ or $Zap70^{mrt/mrt}$ mice

(A) Incidence and representative examples of IgG anti-nuclear or anti-cytoplasmic autoantibodies in 8–15 week old mice of the indicated *Zap70* genotypes. (B) Concentration of serum IgE and IgG1 antibodies in unimmunized mice of the indicated genotypes as measured by ELISA. (C) Relative ELISA titres of specific antibody to T-dependent (*B. pertussis*, CGG, ABA) and T-independent (NP2) immunogens 14 days and 6 days after primary and secondary immunization, respectively. Asterisk indicates P < 0.05.



Figure 3. Graded and threshold effects of different *Zap70* alleles upon peripheral T cell subsets Representative percentage of lymphocytes (A), absolute number (B), and ratio (C) of CD4 and CD8 T cells in spleens of mice with the indicated *Zap70* genotypes. (D) Absolute numbers of CD4⁺ and CD8⁺ lymphocytes within naïve (CD44^{lo}) or activated/memory (CD44^{hi}) compartments. Asterisk indicates *P* <0.05. (E) Anti-CD3-induced calcium flux in CD4 T cells from wild-type and *mrd/mrd* (upper panel) or *mrd/mrt* mice (lower panel).



Figure 4. Four allelic graded states of TCR signaling in thymocytes

Representative percentage of lymphocytes (A), and absolute number (B), of CD4⁻CD8⁻, CD4⁺CD8⁺, CD4⁺CD8⁻ and CD4⁻CD8⁺ thymocytes in B10.Br mice of the indicated *Zap70* genotypes. (C) Normalised mean fluorescent intensities (MFI) of CD5, CD69, TCR β and CD3 ϵ expression upon CD4⁺CD8⁺ thymocytes, where the mean value in wild-type cells represents 100 units. Asterisk indicates *P* <0.05. Representative histograms are shown in Supplementary Figure 1.





(A) Percentage of CD8⁺ or CD4⁺ T cells expressing V β 11⁺ or V β 5⁺ in peripheral blood lymphocytes of nontransgenic mice with the indicated *Zap70* genotypes, and either *H*2^b (open symbols) or *H*2^k (filled) haplotypes. Asterisk indicates *P* <0.05. (B–D) Representative flow cytometry profiles and (B) percentages of thymocyte CD4 and CD8 subsets in TCR transgenic or TCR insHEL double-transgenic mice with wild type *Zap70* or homozygous *Zap70^{mrd/mrd}*. (C) Spleen cells from the same mice as (B), stained for CD4 and the 3A9 TCR clonotype, 1G12. (D) 1G12 clonotype staining on gated CD4⁺CD8⁻ thymocytes from (B) and from a nontransgenic control. Numbers show percentage of CD4⁺CD8⁻ cells, with bracketed numbers indicating percentage of all thymocytes, that are 1G12⁺ or 1G12^{hi}. (E) Absolute number of CD4⁺CD8⁻ 1G12^{hi} cells in the thymus of two animals of each genotype.



Figure 6. Threshold effects of decreased ZAP-70 activity on Foxp3⁺ regulatory T cells (A) Representative flow cytometry profiles of CD4 and Foxp3 expression and percentage of CD4⁺Foxp3⁺ cells in thymocytes or splenocytes from mice of the indicated *Zap70* genotypes. (B) Absolute numbers and percentages of CD4⁺CD8⁻ thymocytes or splenocytes expressing Foxp3. Asterisk indicates P < 0.05. (C) Representative histogram overlay of CD25 expression upon CD4⁺Foxp3⁺ splenocytes from B10.Br (shaded), *mrd/mrd* (dotted), or *mrd/mrt* (solid) mice.



Figure 7. Failure to suppress IgE secretion in *mrd/mrt* chimeric mice with +/+ $CD4^+Foxp3^+$ cells (A) Irradiated *RAG1*-deficient mice were reconstituted with mixtures of fetal liver cells from CD45.2⁺ *mrd/mrt* and CD45.1⁺ wild-type or *Foxp3*-deficient donors, and serum IgE measured 6–7 weeks post-reconstitution. Columns show median values; ns, no significant difference; asterisk, significant *P* <0.05, between indicated experimental group and 100% *mrd/mrt* chimeras by Kruskal-Wallis test with Dunn's post-test. (B) Chimeric mice were analysed at the same time for the percentage of CD4 T cells derived from *Zap70*-wildtype CD45.1 donor stem cells. (C) Flow cytometry profiles of CD45.1 and Foxp3 expression in CD4⁺ splenocytes from representative chimeric recipients. Serum IgE concentrations for each mouse are given alongside.