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Altered Thymic Selection and Increased Autoimmunity Caused by Ectopic Expression of DRAK2 during T Cell Development

Martina Gatzka, Ryan H. Newton, and Craig M. Walsh

Negative regulation of TCR signaling is an important mechanism enforcing immunological self-tolerance to prevent inappropriate activation of T cells and thus the development of autoimmune diseases. The lymphoid-restricted serine/threonine kinase death-associated protein-related apoptotic kinase-2 (DRAK2) raises the TCR activation threshold by targeting TCR-induced calcium mobilization in thymocytes and peripheral T cells and regulates positive thymic selection and peripheral T cell activation. Despite a hypersensitivity of peripheral drak2-deficient T cells, drak2-deficient mice are enigmatically resistant to induced autoimmunity in the model experimental autoimmune encephalomyelitis. To further evaluate the differential role of DRAK2 in central vs peripheral tolerance and to assess its impact on the development of autoimmune diseases, we have generated a transgenic (Tg) mouse strain ectopically expressing DRAK2 via the lck proximal promoter (1017-DRAK2 Tg mice). This transgene led to highest expression levels in double-positive thymocytes that are normally devoid of DRAK2. 1017-DRAK2 Tg mice displayed a reduction of single-positive CD4+ and CD8+ thymocytes in context with diminished negative selection in male HY TCR × 1017-DRAK2 Tg mice as well as peripheral T cell hypersensitivity, enhanced susceptibility to experimental autoimmune encephalomyelitis, and spontaneous autoimmunity. These findings suggest that alteration in thymocyte signaling thresholds impacts the sensitivity of peripheral T cell pools. The Journal of Immunology, 2009, 183: 285–297.

A s adaptive immunity must maintain a highly diverse and specific T cell repertoire to defeat microbial pathogens and cancer cells while preventing aberrant activation by self-Ags, multiple surveillance mechanisms have evolved to ensure immunological self-tolerance. The mechanisms of central (thymic) T cell tolerance—negative selection of immature thymocytes with high affinity for self-peptide-MHC by apoptosis (1, 2) and development of natural regulatory T cells (Tregs) (3)—cooperate with peripheral tolerance mechanisms including anergy induction, clonal deletion by activation-induced cell death (AICD), and suppression by CD4+CD25+ Tregs (4). Because T cell fate at different stages of lymphoid development is determined by the strength, duration, and frequency of signals emanating from the TCR as well as the costimulatory context, TCR signaling is subject to extensive negative and positive feedback regulation to ensure proper responses (5).

Depending on their expression and activation patterns during T cell development, negative regulators of TCR signaling may impact central and/or peripheral tolerance mechanisms. Examples of such negative regulators that act downstream of the TCR include the E3 ubiquitin ligases c-Cbl, Cbl-b, Itch, and GRAIL; phosphatases Sts-1/2, SHP-1, PEP, PTP-PEST; adaptor proteins Dok-1/2; the lipid phosphatase PTEN; and the serine/threonine kinase death-associated protein-related apoptotic kinase-2 (DRAK2) (6). Germline deletion of such genes in mice commonly results in T cell hyperreactivity and, with the exception of DRAK2, increased susceptibility to autoimmune disease. The majority of these negative regulatory proteins support peripheral tolerance by raising the threshold of peripheral T cell activation to enforce the requirement for costimulation, and thereby limit spurious activation by self-Ags, as recently reviewed in Ref. 6. In addition, potential effects on the selection of the T cell repertoire in the thymus either via a direct influence on apoptotic signals or by shifting the threshold to rescue T cells with intermediate or stronger affinity for self-peptide/MHC may be superimposed on any impact on peripheral T cell activation. Negative regulators of TCR signaling that influence thymic selection events include c-Cbl (7), SHP-1 (8), PTEN (9), and DRAK2 (10). The importance of the TCR signaling threshold during thymic selection to peripheral T cell function is underscored by the development of spontaneous polyarthritis in SKG mice that bear a point mutation in the gene encoding the tyrosine kinase ZAP70 (11). Because the SKG mutation diminishes the activity of ZAP70, it is thought that this autoimmune phenotype results from enhanced positive selection of T cells

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3 Address correspondence and reprint requests to Dr. Craig M. Walsh, University of California, Irvine, 3215 McGaugh Hall, Irvine, CA 92697-3900. E-mail address: cwalsh@uci.edu
4 Abbreviations used in this paper: Treg, regulatory T cell; AICD, activation-induced cell death; ANA, anti-nuclear Ab; DAPK, death-associated protein-like kinase; DP, double positive; DRAK2, DAP-related apoptotic kinase-2; EAE, experimental autoimmune encephalomyelitis; HA, hemagglutinin; IGHV-3’ UTR, 3’ untranslated region of human growth hormone; MOG, myelin oligodendrocyte glycoprotein; sPCR, quantitative real-time RT-PCR; SP, single positive; Tg, transgenic.

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exhibiting highly avid TCR, allowing for the escape of autoreactive T cell clones (that should have been deleted) to the periphery.

The serine/threonine kinase DRAK2, also termed STK17b, makes up the fifth member of the death-associated protein-like kinase (DAPK) family and is predominantly expressed in the thymus, lymph nodes, and spleen. The kinase is differentially expressed during both T and B cell development, with highest levels of *drak2* transcript in the most mature lymphocyte populations, namely single-positive (SP) thymocytes, naive peripheral T cells and mature B cells (10). In contrast to the reported ability of DRAK2 to promote apoptosis after ectopic expression in cell lines (12, 13), its germline deletion in mice does not lead to any discernable reduction in apoptotic sensitivity in the lymphoid system. Instead, *drak2*-deficient mice revealed a paradoxical and unique role of the kinase in the regulation of T cell activation and tolerance (10). Despite conferring T cell hyperreactivity to TCR stimulation in vitro with reduced activation threshold and diminished requirement for CD28-mediated costimulation, *drak2* deficiency does not lead to lymphadenopathy or spontaneous autoimmunity in aging mice. Contrarily, loss of *drak2* results in an unexpected resistance to autoimmune disease in a model of EAE induced by injection of myelin oligodendrocyte glycoprotein (MOG) peptides.

During thymic T cell development, *drak2*-deficient mice display slightly increased positive selection of CD4+ T cells and a reduced TCR activation threshold (10, 14). This impact on central tolerance might contribute to the altered responsiveness of peripheral T cells. Because of their hypersensitivity to antigenic stimulation, mature peripheral *drak2*−/− T cells are capable of proliferating in the absence of costimulation via CD28 but develop a large apoptotic population (15), indicating that costimulatory signals are necessary to prevent AICD under such conditions. Although the direct substrates and intracellular targets of DRAK2 kinase activity remain to be identified, experiments have shown that DRAK2 exerts its inhibitory effect on TCR signaling by modulating Ca2+ mobilization as *drak2*-deficient thymocytes and peripheral T cells display an increase in Ca2+ influx upon TCR cross-linking. DRAK2 activity itself is induced by Ca2+ mobilization, demonstrating that it serves in a negative regulatory loop to temper Ca2+ signaling (16). Similar to the endogenous calcineurin inhibitor calcipressin 1 (17), DRAK2 may thus function in a signaling module to suppress the expression of high-threshold genes such as Fas ligand during initial clonal expansion of T cells, thereby preventing premature death under certain conditions such as repetitive or suboptimal stimulation.

To investigate whether DRAK2 impacts thymic selection, the peripheral T cell repertoire and autoimmune susceptibility, we have developed a transgenic (Tg) mouse model in which DRAK2 expression is driven by the *lck* proximal promoter (1017-DRAK2 Tg mice). In such mice, DRAK2 is ectopically expressed at high levels in double-positive (DP) thymocytes, a subset with normally low levels of this inhibitory kinase. We hypothesized that, similar to SKG mice bearing a mutation in the ZAP70 gene, a heightened activation threshold imposed by DRAK2 in developing thymocytes might cause enhanced selection of autoreactive T cells. This hypothesis is based on the premise that with such an enhanced activation threshold, developing thymocytes normally fated to be eliminated by negative selection would instead be rescued and released into the periphery. In contrast to a DRAK2 Tg mouse strain ubiquitously overexpressing DRAK2 from the β-actin promoter (18), Tg expression of DRAK2 from the *lck* proximal promoter leads to T cell lineage- and developmental stage-specific expression. Analysis of T cell subset distribution and function as well as spontaneous and induced autoimmunity in 1017-DRAK2 Tg mice has revealed significant reductions in SP CD4+ and CD8+ thymocytes due to altered positive and negative selection, a hyperreactivity of the resulting peripheral T cell population in context with organ infiltration in aged mice, and an increased susceptibility to EAE. These results demonstrate that DRAK2 enforced negative regulation of TCR signaling during thymocyte development leads to selection of mature T cells with altered activation thresholds for clonal expansion and an increased reactivity toward self-Ags.

Materials and Methods

Generation of 1017-DRAK2 Tg mice

Full-length murine *drak2* cDNA in context with a hemagglutinin (HA)-tag was cloned into the BamHI site of the 1017-*lck* vector containing the Iκκ proximal promoter and the 3’ untranslated region of human growth hormone (hGH-3’ UTR). The NotI fragment of the resulting 1017-*drak2* construct was then injected into fertilized C57BL/6J mouse oocytes. Screening for transgene incorporation and genotyping were performed using a PCR-based approach with primers specific for *drak2* and the hGH-3’ UTR. 1017-DRAK2 Tg mice used for functional experiments have been backcrossed onto the C57BL/6J background for at least five generations and were between 4 and 8 wk of age unless otherwise indicated. In all cases, age-matched littersmates were used as controls. H-Y TCR Tg mice (B10.Cg-Tg 71Vbo N12) were purchased from Taconic Farms and bred onto the 1017-DRAK2 Tg background to assess positive and negative selection. All mice were housed in a pathogen-free environment in accordance with the regulations of the Institutional Animal Care and Use Committee at the University of California (Irvine, CA).

FACS analyses

Abs (conjugated with FITC, PE, PerCP or allophycocyanin) directed against the following surface markers were obtained from eBioscience, BD Pharmingen, or Caltag Laboratories and used in 1/400 dilution to analyze immune cell subsets in single cell suspensions of thymi, spleens, and lymph nodes of 1017-DRAK2 Tg mice: CD3, CD4 (RM4-5), CD8, CD25a (p55/IL-2Ra) (7D4), CD44, CD62L (L-selectin) (MEL-14), CD69 (VEA) (H1.2F3), B220/CD45R (RA3–6B2), H-Y TCR (T3.70), and Vj8 screening panel. Apoptotic fractions were detected by annexin V staining (FITC, PE, and allophycocyanin) (BD Biosciences). To detect DRAK2 by intracellular staining, thymocytes were first surface stained with anti-CD4-allophycocyanin and anti-CD8-PE followed by fixation and permeabilization using the Cytofix/Cytperm kit (BD Biosciences). Subsequently, anti-DRAK2 mAb (Cell Signaling Technologies), anti-hemagglutinin (HA), or isotype control Abs were added for 30 min followed by washing and staining with FITC-conjugated anti-rabbit or anti-mouse Abs. All FACS analyses were performed using a FACSCalibur (BD Biosciences) and CellQuest Software as well as FlowJo Software (Tree Star).

Western blot analysis

Whole-cell extracts were prepared from T cell suspensions using whole-cell extract buffer (0.5% Nonidet P-40) as described previously (19). DRAK2 expression was assessed by Western blotting with an anti-DRAK2 mAb or anti-HA Ab to detect either total DRAK2 or Tg overexpression (all Abs were from Cell signaling Technologies).

Analysis of DRAK2 mRNA expression by quantitative real-time RT-PCR (qPCR)

After isolation of total cellular RNA from either thymocytes or splenocytes with TRIzol solution (Invitrogen) according to the manufacturer’s instructions, cDNA was generated from 1 μg of total RNA using the Superscript First-stand Synthesis System with oligo(dT) primers (Invitrogen). Samples were analyzed in triplicate by qPCR with an iCycler using the IQTM SYBRGreen Supermix (Bio-Rad) and *drak2*-specific primers for 40 amplification cycles. Expression levels were calculated by normalization of data to *β-actin* mRNA expression.
**Thymocyte apoptosis assay**

Thymocytes were plated at a density of 10^6/well and either left untreated or incubated for 24 h with the following inducers of apoptosis: anti-CD3e Ab (1 μg/ml), ionomycin (1 μM), dexamethasone (1 μM), etoposide (10 μM), and anti-Fas/APO-1 mAb (CD95, Jo2; 1 μg/ml) (eBioscience). After incubation, cell viability was assessed by staining with annexin V and CD4 as well as CD8 and subsequent FACS analysis.

\[^{3}H\]Thymidine incorporation and IL-2 production in dose-response assays

Peripheral T cells for the proliferation and function assays were purified from total splenocytes using the Easy-Sep T cell negative selection kit (StemCell Technologies) before plating at a density of 1 × 10^6 T cells/ml. Typical cell purity was >95%. Mouse anti-CD3e Ab (145-2C11) and anti-CD28 Ab (37.51) (both eBioscience) were used for T cell activation as indicated in either plate-bound or soluble form. To assess proliferative capacity, total splenocytes or purified T cells from 1017-DRAK2 Tgs and littermates were plated in triplicate at a density of 1 × 10^5/100 μl in round-bottom 96-well plates and stimulated for 96 h with series of concentrations of soluble or plate-bound or anti-CD3 (2, 0.2, and 0.02 μg/ml) in the presence of absence of anti-CD28. For the last 18 h of culture, cells were pulsed with 1 μCi/ml \[^{3}H\]thymidine (NEN Research Products) and harvested, and \[^{3}H\] incorporation was then quantified as counts per minute using a beta counter. IL-2 levels of supernatants were measured in triplicate by ELISA with an anti-mouse IL-2 Ab pair (JES6-1A12 and JES6-5H4) (eBioscience).

**CFSE proliferation assay**

Purified T cells (or whole splenocytes) were labeled with 5 μM CFSE (Molecular Probes) and subsequently plated at a density of 1 × 10^6/well before stimulation with plate-bound anti-CD3 as described above with or without 1 μg/ml soluble anti-CD28. As CFSE is converted into CFSE by intracellular esterases, CFSE-labeled cells were collected, costained with anti-CD4-allophycocyanin, anti-CD8-PerCP, and annexin V-PE, and analyzed by four-color flow cytometric analysis using a FACS Calibur following the indicated culture periods.

**Calcium mobilization assays/flux**

To assess calcium flux, thymocytes or purified splenic T cells were labeled with the Ca^{2+} indicator dyes Fura-Red (2 μM) and Fluo-3 (1 μM) in the presence of 0.2% pluronic (all Molecular Probes). Ca^{2+} mobilization kinetics were analyzed by flow cytometry after preincubation of labeled cells with biotinylated anti-CD3 and anti-CD4 upon cross-linking with streptavidin in different thymocyte subpopulations like DP thymocytes, SP thymocytes, and transitional stages as well as peripheral T cells and compared with maximal calcium release induced by addition of ionomycin.

**Experimental autoimmune encephalomyelitis (EAE)**

EAE was induced in groups of 8–10-wk-old 1017-DRAK2 Tgs and wild-type littermates as previously described (10, 20) by immunization at day 0 with 125 μg of MOG (MOG35–55) peptide (prepared in the laboratory of Prof. Charles Glabe, University of California, Irvine, CA) emulsified in CFA, containing heat-inactivated H37Ra Mycobacterium tuberculosis (Fisher Scientific), in each hind flank combined with i.p. injection of 200 ng of Bordetella pertussis toxin (List Biologicals) in sterile PBS immediately after immunization and again on day 2. On day 7, a booster immunization with another dose of MOG35–55 in CFA followed by one injection of pertussis toxin was given. To evaluate the impact of DRAK2 overexpression on thymic tolerance/selection at a sensitive stage of thymocyte development where DRAK2 is not expressed yet physiologically, a Nol1 transgenic mouse strain was backcrossed to 129SvEvTac/Crl mice with high copy integration of the transgene. Among these initial founders, two strains (26.1 and 17.2) with high ectopic expression of HA-DRAK2 in thymocytes were selected for further studies. In contrast to the robust overexpression observed in developing thymocytes, expression of HA-DRAK2 in peripheral splenic T cells was only marginally enhanced compared with endogenous DRAK2 protein levels observed in wild-type splenic T cells (Fig. 1B). When fractionated into CD3^+ vs B220^- subsets using magnetic purification, only a small amount of HA-DRAK2 was observed in the former, with no signal observed in the B cell fraction (Fig. 1C); no appreciable increase in total DRAK2 was observed in either splenocyte population. To determine that the thymocyte subpopulation(s) bearing transgene expression, we made use of an intracellular staining protocol using either anti-HA or anti-DRAK2 mAbs. Using this approach, we observed anti-HA staining in a significant fraction of 1017-DRAK2 Tg thymocytes (Fig. 1D), primarily resulting from HA-DRAK2 expression in DP thymocytes (supplemental Fig. 1). Although Western blotting suggested that DRAK2 might be overexpressed in the thymi of 1017-DRAK2 mice, anti-DRAK2 intracellular staining revealed that HA-DRAK2 is instead misexpressed (Fig. 1E). Overall DRAK2 expression was greatly enhanced only in DP thymocytes, a subset that we have previously observed to be mostly devoid of this immunomodulatory kinase (10, 14). DRAK2 expression was only modestly increased in the CD4^+ and CD8^+ SP populations, demonstrating that DRAK2 is ectopically expressed during thymocyte development. The modest increase in total DRAK2 in SP thymocyte subsets was also observed in peripheral CD4^+ and CD8^+ splenocytes. At the mRNA level, a significant increase of drrak mRNA expression over wild-type levels was confirmed by real-time RT-PCR in thymocytes (up to 250-fold), whereas ectopic drrak mRNA expression in total peripheral splenocytes was only minimal (~4-fold) in these 1017-DRAK2 Tg lines (Fig. 1G). Offspring from these two Tg founders were backcrossed at least five generations onto the C57BL/6J-background, expanded, and used for detailed studies described below. All 1017-DRAK2 Tg mice were born at Mendelian ratios, viable, fertile, and had no gross abnormalities upon visual inspection (data not shown).
Thymi, spleens, and lymph nodes of Tg and wild-type littermates were harvested at various ages (4–6 and 10 wk as well as 12 mo for aging experiments) and assessed for organ size, weight, total cellularity, as well as distributions of various immune and T cell populations. A subfraction of 1017-DRAK2 Tg mice displayed splenomegaly early in life (4–6 wk of age), and most mice developed a splenomegaly with age (see below). Whereas the numbers of total thymocytes were overtly normal in 1017-DRAK2 Tg mice, a moderate but consistent increase in spleen weight and total cellularity was detectable (Fig. 2A); the number of total splenic T cells after purification was also slightly increased in the Tg mice. The most striking result was a significant and consistent reduction of the percentages of SP CD4⁺/His and CD8⁺/H11001 T cells and a concomitant increase of the DP population in the thymi of young 1017-DRAK2 mice (Fig. 2B), potentially reflecting impaired positive selection. The decrease in SP CD4⁺ and CD8⁺ T cells was accompanied by reduced numbers of SP CD5high/CD69low and HSAlow/CD3high thymocytes in DRAK2 Tg mice, indicating that the intensity of the signal through the TCR was muted during thymocyte selection (Fig. 2C). No obvious differences were observed in double-negative thymocyte subsets, as assessed by costaining of CD4/CD8 T cells with anti-CD25 and -CD44 (data not shown). In the periphery, 1017-DRAK2 Tg mice displayed overtly normal ratios of T and B cells as verified by staining with anti-CD3 and anti-B220 (Fig. 2D). Strikingly, the CD4⁺ subset expressing CD25 was embellished in all DRAK2 Tg mice examined without concomitant up-regulation of the transient activation marker CD69 (Fig. 2E). In accordance, intracellular staining with an Ab against the transcription factor Foxp3 that serves as a specific marker for the Treg subset revealed elevated levels of Foxp3, confirming that these CD4⁺/CD25⁺ T cells possess...
features of Tregs (data not shown). In contrast to a previous report in which DRAK2 expression is mediated by the β-actin promoter (18), the fraction of CD4^+ T cells with an activated or memory T cell phenotype (CD44^high/CD62L^low) did not appear to be decreased in 1017-DRAK2 Tg mice. Contrarily, an increase in this T cell population was detectable in most 1017-DRAK2 Tg mice, potentially resulting from activation of peripheral T cells by environmental or self-Ags (see below).

**Alterations in positive and negative thymic selection in H-Y 1017-DRAK2 mice**

To further explore the potential that ectopic DRAK2 expression limits thymic selection, 1017-DRAK2 Tg mice were crossed onto the H-Y TCR Tg background. H-Y TCR Tg (H-Y) mice express a TCR specific for the male H-Y Ag presented in the context of H-2Db molecule. Thymocytes bearing the Tg TCR are positively selected in females but deleted by negative selection in male mice (23, 24). After crossing 1017-DRAK2 and H-Y TCR Tg mice, thymocytes from female and male H-Y TCR × 1017-DRAK2 double Tg mice (H-Y 1017-DRAK2) were analyzed by FACS after staining with anti-CD4, anti-CD8, and clonotypic T3.70 anti-TCR Abs to compare the ratios of DP to SP CD8^+ T cells as well as the level of Tg TCR expression (H-Y TCR^{high} vs TCR^{low}). In female H-Y mice, mature CD4^-CD8^+ T cells predominated over CD4^+CD8^- T cells in the presence of a selecting H-2D^b haplotype in the C57BL/6J, as expected (Fig. 3A). This otherwise augmented proportion of SP CD8^- T cells (average 16%) was significantly reduced in H-Y 1017-DRAK2 female mice (average 8%) reflecting impaired positive selection. Male H-Y mice normally have very small thymi due to deletion of the self-reactive T cells, and thymic sizes and thymocyte numbers were also reduced in H-Y 1017-DRAK2 male mice (data not shown). Despite this, we observed an increased proportion of CD4^-CD8^+ T cells bearing the H-Y clonotypic TCR (Fig. 3B). In the periphery, similar proportions of T3.70^+CD4^- and CD8^- T cells were observed (data not shown). We conclude that ectopic DRAK2 intensified negative regulation of signals through the TCR in developing thymocytes, leading to partial blockade of thymic positive and negative selection.

**Normal apoptotic sensitivity but altered TCR signaling in 1017-DRAK2 Tg mice**

As ectopic expression of DRAK2 in cell lines has previously been shown to correlate with increased apoptosis (12, 13),...
1017-DRAK2 thymocytes were tested for their apoptotic sensitivity following treatment under a variety of conditions. No increased spontaneous apoptosis or altered sensitivity to apoptogenic stimuli could be detected by annexin V staining, and cell recovery was grossly similar to control thymocytes (Fig. 4A). To investigate whether the changes in thymic selection detected in 1017-DRAK2 Tg mice translate into altered function of mature peripheral T cells, we subsequently examined T cell activation to optimal and suboptimal stimuli in dose-response assays assessing proliferation, apoptotic sensitivity, and IL-2 production. Our data indicated that unpurified and purified 1017-DRAK2 T cells hyperproliferated in vitro in response to suboptimal concentrations of anti-CD3 (±anti CD28), as measured by [3H]thymidine incorporation (Fig. 4B). To distinguish between increased cell division rates and reduced apoptosis, proliferation and apoptosis of CD4⁺ and CD8⁺ T cells were quantified simultaneously in the same cultures using CFSE-labeling and annexin V staining after 72 h of stimulation in similar dose-response assays with plate-bound anti-CD3 stimulation as described above (Fig. 4C). Proliferation in response to suboptimal doses of anti-CD3 was enhanced but was not a consequence of diminished apoptosis of proliferating 1017-DRAK2 T cells because the proportion of annexin V⁺ cells was similar or slightly increased compared with controls (supplemental Fig. 2). Consistent with a diminished activation threshold for proliferation, purified 1017-DRAK2 T cells also produced elevated levels of IL-2 in response to suboptimal stimulation as measured by ELISA after 24 h of culture (Fig. 4D).

**Reduced calcium mobilization in developing 1017-DRAK2 DP thymocytes but not in mature SP thymocytes**

Since previous studies have demonstrated that endogenous DRAK2 negatively regulates TCR-induced Ca²⁺ mobilization in transitional and SP thymocytes (14), we sought to evaluate the impact of ectopic DRAK2 expression on this signaling cascade. Thymocytes labeled with fluorescently tagged anti-CD4 and anti-CD8 as well as the Ca²⁺ indicator dyes Fluo-3 and Fura-Red were analyzed by flow cytometry upon TCR/CD4 cross-linking with streptavidin after incubation with biotinylated anti-CD3 and anti-CD4 (Fig. 5A). In thymocytes, the overall diminished Ca²⁺ mobilization in response to anti-CD3/anti-CD4 cross-linking reflected a strong reduction in the DP thymocyte population that expresses the highest amounts of the DRAK2 transgene (see above). In contrast, mature SP CD4⁺ thymocytes displayed grossly similar Ca²⁺ mobilization kinetics as controls. These results indicate a correlation of DRAK2 transgene expression level and TCR-induced calcium signaling threshold. Maximal Ca²⁺ release induced by ionomycin was comparable between 1017-DRAK2 and wild-type cells in all populations analyzed. In contrast, ectopic DRAK2 expression in the thymus did not affect other signaling pathways induced by CD3/CD4 cross-linking; no differences in Erk, Jnk, or tyrosine phosphorylation were observed in 1017-DRAK2 Tg thymocytes after stimulation with increasing concentrations of anti-CD3 (plus constant anti-CD4) (Fig. 5B). In line with the relatively normal Ca²⁺ mobilization of 1017-DRAK2 CD4⁺ SP thymocytes, peripheral CD4⁺ and CD8⁺ T cell Ca²⁺ mobilization after anti-CD3 cross-linking was indistinguishable between wild-type and Tg T cells (Fig. 5C). Remarkably, we observed consistently enhanced basal Erk and tyrosine phosphorylation in 1017-DRAK2 Tg peripheral T cells (Fig. 5D). As well, treatment with a suboptimal dose (0.3 µg/ml) of anti-CD3 induced strong Erk and tyrosine phosphorylation in 1017-DRAK2 T cells, whereas this treatment failed to do so in wild-type T cells. We note that despite these signaling differences, no appreciable differences in the expression of CD3 were observed in 1017-DRAK2 thymocytes (supplemental Fig. 3A). Also, a significant fraction of peripheral T cells had slightly but consistently reduced CD3 or TCRβ-chain expression (supplemental Fig. 3b), demonstrating that the enhanced signaling of these cells is not due to embellished expression of CD3 or TCR molecules on the surface of 1017-DRAK2 T cells. Our data thus indicate that ectopic DRAK2 in the thymus negatively influences calcium-signaling pathways downstream of the TCR. This artificially high activation threshold, as revealed under strong TCR cross-linking conditions (supplemental Fig. 4), leads to the development or selection of T cells with enhanced signaling properties, likely a result of altered selection in the thymus because DRAK2 expression is relatively normal in Tg peripheral T cells.

**Splenomegaly and enhanced susceptibility to spontaneous and induced autoimmunity in 1017-DRAK2 Tg mice**

To assess the manifestation of spontaneous autoimmunity, groups of 1017-DRAK2 Tg mice and littermates were aged for 12 mo and examined for the presence of cellular infiltrates in their major organs by routine histology. In addition, amounts of...
FIGURE 4. Functional analysis of 1017-DRAK2 Tg T cells revealed a hyperresponsiveness of peripheral T cells in response to suboptimal stimulation but no significant changes in the apoptotic sensitivity of thymocytes and peripheral T cells. To assess proliferation and IL-2 production, total splenocytes or purified T cells from either DRAK2 Tg or wild-type mice were seeded in triplicate for each condition at a density of 10^6/ml and stimulated with indicated amounts of plate-bound or soluble anti-CD3 in the presence or absence of anti-CD28 (1 μg/ml) for the times indicated. Data represent averages of at least three individual experiments.

A, Apoptosis is not increased in 1017-DRAK2 Tg thymocytes as assessed in a thymocyte apoptosis assay after in vitro incubation under various conditions for 24 h as indicated. The following concentrations were used: untreated (no), anti-CD3 Ab (2C11) (1 μg/ml), ionomycin (1 μM), anti-Fas mAb (1 μg/ml), dexamethasone (dex; 1 μM), and etoposide (eto; 10 μM).

B, [3H]Thymidine uptake. Hyperproliferation of DRAK2 Tg total splenocytes and purified T cells in response to suboptimal stimulation with plate-bound anti-CD3 in the presence or absence of anti-CD3 in vitro (dose-response assay) (n > 9). Proliferating cells were pulsed with [3H]thymidine during the last 18 h of a 96-h incubation period, and tritium incorporation was measured as counts per minute using a beta scintillation counter.

C, CFSE dilution assay to simultaneously monitor proliferation and apoptosis. Purified T cells from either wild-type (Wt) littermates (solid gray lines) or DRAK2 Tgs (dashed black lines) were labeled with CFSE as described, stimulated with indicated concentrations of plate-bound anti-CD3 for 72 h, and then stained with anti-CD4, anti-CD8, and annexin V for FACS analysis. Increased proliferation of DRAK2 Tg T cells as indicated by higher fractions of cells with more diluted CFSE was not due to a reduction of apoptosis.

D, Increased IL-2 production of peripheral DRAK2 Tg T cells in response to suboptimal stimulation as measured by ELISA in culture supernatants after 24 h of stimulation.
FIGURE 5. Reduced calcium flux in DP thymocytes from 1017-DRAK2 Tg mice stimulated with anti-CD3 and anti-CD4 but not in SP thymocytes. A, To detect alteration in calcium signaling upon TCR stimulation by flow cytometry, thymocytes labeled with Fluo-3 and Fura red and stained with CD4 and CD8 were incubated with biotinylated Abs to CD3 and CD4 before cross-linking with streptavidin as described. Black lines indicate calcium flux in DRAK2 Tg thymocytes, and gray lines represent wild-type controls. For FACS analysis, thymocytes were first sampled for ~60 s to establish baseline Ca^{2+} levels before stimulation with streptavidin (arrow “SA”). After ~480 s of stimulation, maximal calcium release was induced by addition of ionomycin (arrow “iono”). Data represent the kinetics of calcium mobilization in response to stimulation in total thymocytes, DP thymocytes, and CD4^{+} thymocytes plotted as ratio of FL-1 (Fluo-3) to FL-3 (Fura red). B, Unaltered signaling in 1017-DRAK2 Tg thymocytes in response to TCR cross-linking. Total thymocytes were incubated with the indicated amounts of biotinylated anti-CD3 plus biotinylated anti-CD4, followed by cross-linking for 2 min with streptavidin. Western blotting was performed on lysates using anti-phospho- and total-Erk1/2, anti-phospho- and total-Jnk1/2, anti-phospho-tyrosine, anti-DRAK2, and anti- \(\beta\)-tubulin as a loading control. C, Detection of calcium signaling as performed in A on total splenocytes, except that incubation was with anti-CD3 alone before cross-linking with streptavidin as described previously. D, Altered signaling in 1017-DRAK2 Tg splenocytes in response to TCR cross-linking. Total splenocytes were incubated with the indicated amounts of biotinylated anti-CD3 \pm biotinylated anti-CD28, followed by cross-linking for 2 min with streptavidin. Western blotting was performed as in B.
ANA were measured in sera from these aged mice. Splenomegaly was detectable in a subfraction of young 1017-DRAK2 Tg mice, and five of six aged Tg mice displayed overt splenomegaly, with an altered histological architecture and hyperplasia of the red and white pulp. In contrast, only one wild-type mouse developed increased spleen size. A representative example of this degree of splenomegaly in 1017-DRAK2 Tg mice is shown (Fig. 6A). Mononuclear infiltrates were detected in livers and pancreata of all 1017-DRAK2 Tg mice, while no signs of infiltration were apparent in wild-type littermates (Fig. 6B). Five of six 1017-DRAK2 Tg mice also displayed slightly elevated levels of ANA as determined by ELISA (Fig. 6C). These results suggest an increased prevalence of spontaneous autoimmunity in aged 1017-DRAK2 Tg mice, likely caused by activation of autoreactive T cells that may have escaped thymic negative selection, and/or possess diminished activation thresholds. Consistent with either scenario, we observed a dramatic increase in the proportion of CD44+CD62Llow memory cells in 1017-DRAK2 Tg splenocytes (representative spleens from 12-wk-old littermates). E and F, Altered Vβ repertoire in CD8+ SP splenocytes (F) but not CD4+ SP splenocytes (E) in 8–12-wk-old 1017-DRAK2 Tg mice compared with littermate controls. Total splenocytes were stained with CD4, CD8, and the indicated Vβ chains and analyzed by flow cytometry.

**FIGURE 6.** Increased spontaneous autoimmunity in aged 1017-DRAK2 Tg mice. A, Significant splenomegaly in aged 1017-DRAK2 Tgs (representative spleens). B, Infiltrates in livers of 12-mo-old 1017-DRAK2 Tg mice detected by H&E staining (representative data). C, Sera of aged DRAK2 Tg mice were analyzed for the presence of ANA by routine ELISA. The mean OD450 was slightly increased compared with wild-type (Wt) levels in five of six DRAK2 Tgs. D, Significantly increased CD44+CD62Llow memory cells in 1017-DRAK2 Tg splenocytes (representative spleens from 12-wk-old littermates). E and F, Altered Vβ repertoire in CD8+ SP splenocytes (F) but not CD4+ SP splenocytes (E) in 8–12-wk-old 1017-DRAK2 Tg mice compared with littermate controls. Total splenocytes were stained with CD4, CD8, and the indicated Vβ chains and analyzed by flow cytometry.
The susceptibility of 1017-DRAK2 Tg mice to induced autoimmune disease in the EAE model was tested in the multiple sclerosis model EAE, one of the best-characterized murine models of organ-specific autoimmunity (25). Mice were immunized with a peptide containing residues 35–55 from MOG emulsified in CFA and were evaluated for clinical manifestation of EAE as described previously (10, 20). The mean clinical severity of the EAE was exacerbated in 1017-DRAK2 Tg mice compared with wild-type littermates, as reflected by the average clinical scores (Fig. 7A). In addition, 1017-DRAK2 Tg mice displayed an earlier onset of disease (average of the day when each mouse first scored one) and a greater number of Tgs exhibited profound signs of disease, scoring ≥2 (incidence) (Fig. 7B). Infiltrate density in the CNS correlated with clinical scores in 1017-DRAK2 Tg mice as well as wild-type controls at day 21 postimmunization (data not shown). In light of the phenotypic changes detected in the thymus of 1017-DRAK2 Tgs, it is possible that altered thymic selection resulted in a shift in the generated mature T cell pool. Such T cells would possess an increased affinity and/or avidity to foreign and self-Ags (and/or with altered signaling properties and gene expression profiles), leading to an enhanced sensitivity to autoimmunity.

**Discussion**

1017-DRAK2 Tg mice serve as a model to examine the effect of overexpression of a negative regulator of immunoreceptor signaling in developing T cells, specifically at the DP thymocyte stage that is critical for positive and negative selection. The major findings of the present survey of these newly generated 1017-DRAK2 Tg mice are as follows: 1) reduced generation of SP CD4⁺ and CD8⁺ thymocytes that correlated with changes in positive and negative selection in the H-Y TCR Tg model; 2) hyperresponsiveness of peripheral T cells to suboptimal anti-CD3 stimulation reflected by hyperproliferation and increased IL-2 production, as well as alterations in the composition of the peripheral T cell pool; and 3) enhanced susceptibility to spontaneous and induced autoimmunity. We conclude that strong suppression of TCR signals during thymic selection caused by ectopic DRAK2 expression alters the responsiveness of peripheral T cells. Although this likely may lead to an altered TCR repertoire, our results also demonstrate that T cells that are capable of overcoming this artificially high selection threshold in the thymus possess substantially enhanced signaling properties in the periphery. The resulting enhanced autoimmune susceptibility of the mice is likely a consequence of this diminished negative selection and of the reduced activation threshold of the cells.

As expression of the DRAK2 transgene was only marginally increased in the periphery compared with its high levels in DP thymocytes, the changes in peripheral T cell reactivity observed can likely be attributed to altered central tolerance in 1017-DRAK2 Tg mice. This conclusion is supported by the observation of an enlarged population of T3.70⁺ CD8⁺ T cells observed in H-Y 1017-DRAK2 male mice. In addition, mature 1017-DRAK2 T cells possess considerably diminished levels of HA-DRAK2 compared with thymocytes. Thus, it is tenable that only those thymocytes possessing intrinsically enhanced signaling overcome this artificially high activation threshold. By virtue of diminished HA-DRAK2 expression in peripheral T cells, where the lck proximal promoter is known to be quenched, this high

**FIGURE 7.** Increased susceptibility of 1017-DRAK2 Tg mice to induced autoimmune disease in the EAE model. EAE was induced in DRAK2 Tg mice by immunization with MOG peptide emulsified in CFA as described and compared with responses of wild-type littermates. Severity of EAE was assessed daily for a period of 30 days by a standard scoring system (0–4): 0, no signs of disease; 0.5, altered gait and/or hunched appearance; 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; and 4, complete hind limb paralysis and partial forelimb paralysis. The experiment was performed twice, and data were pooled together. A, Mean EAE clinical scores were significantly increased in DRAK2 Tg mice. Data represent average clinical scores for nine DRAK2 Tg mice and eight wild-type (Wt) littermates. B, DRAK2 Tg mice displayed a significantly increased incidence of EAE in two independent experimental sets, as well as significantly exacerbated mean severity (p = 0.04) and earlier mean onset (p = 0.014) compared with Wt littermates. Statistically significant results indicated with ∗.
activation barrier is removed—a scenario that could contribute to the peripheral hyperreactivity. Consistently, positive thymic selection has been associated with the development of an increased activation threshold to the selecting self-Ag within the thymus to ensure unresponsiveness of the mature T cell in the periphery (26). A critical role for DRAK2 in thymic positive selection has previously been suggested, because drak2-deficient thymi display an increased fraction of CD4+ SP thymocytes with concomitant up-regulation of CD5 and CD69, indicating enhanced signal intensity downstream of the TCR (10, 14). These changes in central tolerance may contribute to the T cell hypersensitivity, increased fraction of memory-like circulating T cells, and the autoimmune resistance in drak2-deficient mice. Our data from 1017-DRAK2 Tg mice confirm that DRAK2 impacts thymic selection and that its enforced expression partially blocks positive and negative selection, likely resulting in the selection of autoreactive T cells. DRAK2 expression is tightly regulated in a stage-specific fashion during thymopoiesis (10). Furthermore, it is a primary response gene in DP thymocytes following TCR and coreceptor cross-linking (14). The results here suggest that this tight regulation of DRAK2 expression is necessary for proper positive selection and central tolerance.

The manifestation of T cell-mediated autoimmunity is currently thought to be influenced by a combination of TCR avidity to autoantigens, negative regulation of signaling, concentration of self-peptide-MHC complexes presented on APC, and the co-stimulatory/inflammatory context (27). Furthermore, the distinct signaling thresholds necessary to induce positive selection (survival), negative selection (apoptosis), or peripheral selection/elimination also impact immunological tolerance (26). During thymic T cell development, only DP thymocytes possessing a TCR with low affinity/low avidity for self-peptide-MHC presented by thymic epithelial cells in the cortex receive a sufficient survival signal to be rescued from death by neglect and subsequently undergo positive selection (1, 2). Subsequent negative selection via apoptosis ensures the intrathymic clonal deletion of immature self-reactive thymocytes with high affinity/low avidity for peripheral tissue-restricted self-Ags ectopically expressed in medullary thymic stromal APC via the transcription factor AIRE (autoimmune regulator) (28–30). Clonal deletion (negative selection) in response to strong signals is mediated by direct activation of the proapoptotic BH3-only protein Bim as well as transcriptional up-regulation of Bim and other proapoptotic genes (31, 32). Since the altered selection pattern detected on the H-Y TCR Tg background did not correlate with enhanced apoptotic sensitivity in 1017-DRAK2 Tg mice, DRAK2 does not appear to directly influence the apoptotic pathways involved in thymic development and likely transduces nonapoptotic signals during thymocyte differentiation.

We propose that excessive negative regulation of TCR signaling by ectopically expressed DRAK2 shifts the threshold for thymic positive and negative selection. Consequently, its hyperexpression may block positive selection, potentially resulting in death by neglect of a population with low avidity for MHC plus self-peptides. On the other hand, it may also rescue thymocytes with a higher affinity/avidity for self-Ag that would otherwise undergo negative selection. Therefore, the resulting peripheral T cell pool in 1017-DRAK2 Tg mice may possess an altered TCR repertoire skewed toward self-reactivity and equipped with a diminished activation threshold. In accord with this model, negative regulation of TCR signaling has been implicated in “tuning” T cell responses to allow high-avidity/low-affinity, self-reactive T cells to avoid negative selection in the thymus (32). Consequently, negative regulation provides non-deleting mechanisms to control the avidity with which T cells recognize self-Ags (“tuning”) to maximize the peripheral T cell repertoire, allowing for survival of T cells that can respond to self, but only at concentrations that are normally not reached in vivo (33). This hypothesis is also supported in models such as SKG mice (11), as well as in the observation that CD28 costimulation can enhance thymic negative selection for certain Vß subsets (34–36). Furthermore, inhibition of miR-181a expression in immature T cells has recently been demonstrated to reduce intrinsic Ag sensitivity and impair both positive and negative selection via up-regulation of multiple phosphatases (37). Taken together, these findings suggest that activation threshold must be carefully tuned to achieve appropriate responsiveness of peripheral T cell pools.

TCR-induced signaling pathways that mediate positive and negative selection by regulating the balance of pro-/antiapoptotic factors and apoptotic sensitivity in thymocytes include Ca2+ influx, calcineurin-NFAT-, MAPK-, and protein kinase C pathways. Notably, modulation of signal strength and duration likely determines cell fate, with high-intensity signals required to promote execution of cell death and low-intensity stimuli driving positive selection (33). A function of DRAK2 in controlling the threshold for calcium mobilization in the thymus and peripheral T cells has previously been established, since positively selected drak2-deficient thymocytes as well as mature peripheral T cells display enhanced Ca2+ mobilization after suboptimal TCR cross-linking (10, 14). In accord with the notion that DRAK2 sets the threshold for Ca2+ mobilization in developing thymocytes, we observed reduced Ca2+ mobilization in 1017-DRAK2 DP thymocytes (but normal MAPK activation and tyrosine phosphorylation), cells that express the highest levels of the transgene, but not in SP thymocytes and peripheral T cells that have modest levels of transgene expression. Recent data indicate that calcium signals are necessary for the induction of positive as well as negative selection: strong TCR-signals lead to Ca2+-dependent transcriptional induction of pro-apoptotic Bim via protein kinase C activation (38). In contrast, weaker signals activate the Ca2+-NFAT pathway to mediate positive selection of thymocytes via MAPK/Erk sensitization (39). Consistently, T cell-specific deletion of NFATc3 or calcineurin in mice causes defects in positive selection (40). The defect in Ca2+ mobilization detected in 1017-DRAK2 DP thymocytes may thus explain the effects of this transgene on positive and negative selection. However, a more complete understanding of the consequences of ectopic DRAK2 expression during thymic selection awaits full description of its kinase substrates in thymocytes.

In Tg mice expressing DRAK2 via the β-actin promoter (18), a careful analysis of thymic development has not been described, nor have expression patterns of the transgene in thymocyte and other immune subsets been examined. Instead, that study has focused entirely on peripheral T cells. Because of ubiquitous overexpression of DRAK2 in that strain, changes in multiple immune and accessory cells could potentially impact T cell responses and contribute to the phenotype observed. Similar to our results with 1017-DRAK2 Tgs, this previous report described hyperresponsiveness of peripheral T cells, with hyperproliferation and augmented production of IL-2 (and IL-4) in response to anti-CD3 stimulation. In contrast to our findings described here, Mao et al. (18) also described increases in peripheral T cell apoptosis mediated by IL-2 and a defect in memory cell development. Although 1017-DRAK2 peripheral T cells produce elevated amounts of IL-2, no major increases in
apoptosis were detectable in response to anti-CD3 stimulation. Memory T cell responses in 1017-DRAK2 Tg mice are currently under investigation, but we detected an increase, rather than a reduction, in the fraction of the activated or memory type T cell subset (CD44hi/CD62Llow) in Tg mice up to 14 wk of age. That DRAK2 is critical for memory T cell function has also been established by previous analysis of antiviral responses to murine hepatitis virus (MHV) in drack2-deficient mice (41).

The shift in TCR signaling threshold and peripheral repertoire likely accounts for the enhanced susceptibility of 1017-DRAK2 Tg mice to spontaneous and induced autoimmunity. As discussed above, elevated responses of 1017-DRAK2 peripheral T cells to suboptimal anti-CD3 may represent an indirect effect due to an altered threshold acquired in the course of positive selection with high levels of this negative regulator of TCR signaling. To overcome the higher peripheral activation threshold, costimulation via CD28 normally serves as an amplifier to quantitatively and qualitatively support the TCR signal to induce a proliferative response while preventing anergy and AICD (42); such costimulation is dispensable for the activation of drack2-deficient T cells (10). T cells in 1017-DRAK2 Tg mice that have the potential to recognize self-Ag might be activated more readily, resulting in enhanced spontaneous and induced autoimmune susceptibility. The spontaneous autoimmune hallmarks detected in DRAK2 Tgds have characteristics of a lupus-like disease, being marked by splenomegaly, liver infiltration, and increased ANA (43).

The 1017-DRAK2 Tg mouse model has confirmed that expression of negative regulators of TCR signaling during thymic development may significantly impact central tolerance and the peripheral T cell repertoire by shifting the thresholds for positive and negative selection. Such a shift in TCR repertoire to higher avidity may allow for survival of T cells with intermediate or high affinity/avidity for self-peptide/MHC that may provoke autoimmunity. Taken together, these results suggest that therapeutic efforts to interfere with T cell signaling must be taken with caution to prevent the export of highly autoreactive clones from the thymus.

Acknowledgments

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Supp. Fig. 1: Intracellular staining of 1017-DRAK2 thymocytes with anti-HA mAbs reveals highest differential expression in the double-positive subset. Wildtype (WT) or 1017-DRAK2 transgenic (DTg) thymocytes were harvested and stained with anti-CD4 and anti-CD8, followed by fixation, permeabilization, and staining with anti-HA mAbs.
Supp. Fig. 2: Cell cycle progression vs. apoptosis of 1017-DRAK2 vs. wildtype splenic T cells. Top panel: histograms of CFSE dilution of live-gated CD4 and CD8 T cells of indicated genotype following 4d of stimulation with anti-CD3 +/- anti-CD28 as indicated. Bottom panel: Annexin-V vs. CFSE analyses of non-livegated samples as in top panels. Numbers in upper left quadrants represent the ratio of CFSE diluted Annexin-V<sup>Hi</sup> vs. Annexin-V<sup>Lo</sup> cells.
Supp. Fig. 3: CD3 and TCR expression on 1017-DRAK2 vs. wildtype thymocytes and splenic T cells.  
A Similar surface CD3 levels between wildtype and 1017-DRAK2 thymocyte subsets.  
B Comparison of CD3 and TCRβ surface expression on wildtype vs. 1017-DRAK2 splenic T cell subsets.
Supp. Fig. 4: Calcium mobilization in thymocyte subsets responding to low (0.2 μg/ml) vs. high (1.0 μg/ml) anti-CD3 stimulation.
**Supp. Figure 5**

Supp. Fig. 5: Vβ usage in CD4 vs. CD8 splenocyte T cell subsets from young (4-8 week old) 1017-DRAK2 vs. wildtype mice. Mean % expression +/- SEM presented.