

A Deficiency in *Drak2* Results in a T Cell Hypersensitivity and an Unexpected Resistance to Autoimmunity

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Summary

DRAK2 is a member of the death-associated protein (DAP)-like family of serine/threonine kinases. Members of this family induce apoptosis in various cell types. DRAK2, in particular, is specifically expressed in T cells and B cells, and it is differentially regulated during T cell development. To determine whether DRAK2 regulates lymphocyte apoptosis, we produced *Drak2*^{-/-} mice. Contrary to our expectations, *Drak2*^{-/-} T cells did not demonstrate any defects in apoptosis or negative selection; however, T cells from *Drak2*^{-/-} mice exhibited enhanced sensitivity to T cell receptor-mediated stimulation with a reduced requirement for costimulation. These results provide evidence that DRAK2 raises the threshold for T cell activation by negatively regulating signals through the TCR. In contrast to other models of T cell hypersensitivity, *Drak2*^{-/-} mice were remarkably resistant to experimental autoimmune encephalomyelitis (EAE). These results expose a new pathway regulating T cell activation and highlight the intricacies of induced autoimmune disease.

Introduction

The acquired immune system of vertebrates evolved in response to relentless pressure applied by coevolving infectious agents (Hedrick, 2004). It is unique in exhibiting unlimited potential for molecular recognition as well as a diverse means for inducing cellular destruction. In fact, it is the only physiological system in animals capable of actively executing the loss of organismal integrity. Consequently, numerous mechanisms have evolved that regulate the activation of an immune response and limit its extent and duration. This is particularly important at the level of T cell activation. Regulatory molecules expressed in T cells participate in maintaining cellular quiescence, setting the threshold for activation and limiting the magnitude of a response through negative feedback control (Veillette et al., 2002). Defects in these molecules result in T cell hypersensitivity and often contribute to an increased propensity for autoimmune dis-

ease. Examples include phosphatases such as SHP-1, PEP, PTP-Pest, CD45, SHIP-1, and PTEN; the E3 ubiquitin ligases Cbl-b, c-Cbl, Itch, and GRAIL; inhibitory cell surface receptors CTLA-4 and PD-1; and transcription factors Foxj1, Foxp3, and LKLF (Anandasabapathy et al., 2003; Bachmaier et al., 2000; Brunkow et al., 2001; Chiang et al., 2000; Clark et al., 1999; Coyle and Gutierrez-Ramos, 2001; Fang et al., 2002; Kuo et al., 1997; Lin et al., 2004).

T cell apoptosis is another critical component of tolerance and plays an essential role at two stages in the natural life of a T cell. Developing thymocytes are induced to die as a consequence of high reactivity for self-MHC and peptide complexes, thus reducing the number of potentially autoreactive T cells that progress through development. Apoptosis is also an essential feature of the waning phase of the immune response to an infectious agent.

The signal transduction events leading to apoptosis are well studied and, unlike most other pathways, rarely involve protein phosphorylation. An exception may be apoptosis promoted by the DAP-like family of kinases. Members of this family include DAP kinase, DRP-1, ZIP kinase, DRAK1, and DRAK2 (Kogel et al., 2001). These serine/threonine kinases share homology within their N-terminal kinase domains but vary extensively in their C-terminal domains. All members of this family induced apoptosis after ectopic expression in cell lines (Cohen et al., 1997; Kawai et al., 1999; Sanjo et al., 1998). DAP kinase-related apoptosis-inducing protein kinase 1 (DRAK1) and DRAK2 were identified by EST homology searches for genes containing kinase domains similar to DAP kinase. In addition to its putative role in apoptosis, DRAK is of particular interest because it is expressed most abundantly in lymphoid organs. In fact, an analysis of gene expression patterns in 46 different human tissues revealed that DRAK (also referred to as STK17) is one of only 31 genes expressed exclusively in lymphoid tissue (Su et al., 2002).

Given that apoptosis is so critical for maintaining a well-regulated immune system and that members of the DAP-like kinase family induce apoptosis, we investigated the role of DRAK2 in lymphoid cells. In extensive studies of *Drak2*^{-/-} mice, we found no evidence for an alteration in the sensitivity of thymocytes or mature T cells to apoptosis. Instead, we found that T cells deficient in *Drak2* exhibited increased sensitivity to TCR signals with a reduced requirement for costimulation. From these studies we conclude that DRAK2 functions as yet another negative regulator of T cell activation. Surprisingly, despite this T cell hypersensitivity, *Drak2*^{-/-} mice were highly resistant to EAE, an unexpected result in light of other models of T cell hypersensitivity.

Results

DRAK2 Expression Is Highest in Lymphoid Organs

A query of the Gene Expression Atlas created by the Genomics Institute of the Novartis Research Foundation

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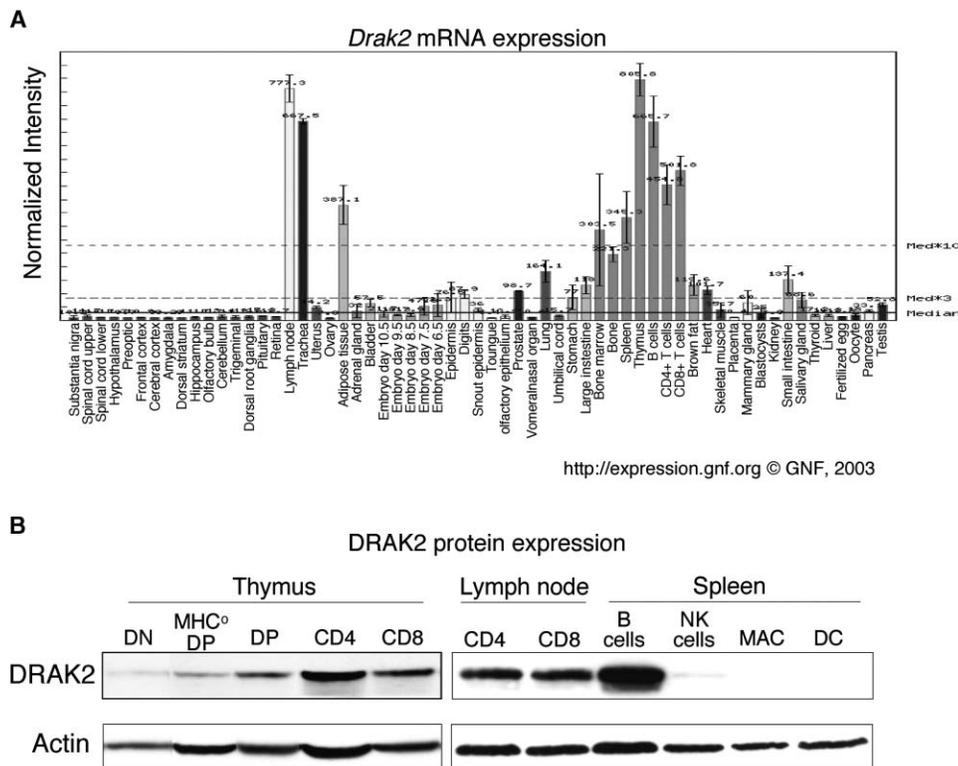


Figure 1. Analysis of DRAK2 Expression

(A) Expression profile of *Drak2* mRNA in mouse tissue. Each bar represents the normalized intensity of the amount of *Drak2* transcript in each tissue.

(B) The various subsets of cells were purified from thymus, spleen, and lymph nodes and analyzed by Western blot for DRAK2 expression. All subsets were greater than 94% pure. For a loading control, the blots were stripped and then blotted with an anti-actin antibody. These data are representative of two separate sorts.

(Su et al., 2002) for *Drak2* expression in mouse tissues demonstrated that like the pattern in human tissues, *Drak2* mRNA expression is highest in lymphoid organs (Figure 1A). To confirm that DRAK2 protein retained the same expression pattern as *Drak2* mRNA and to further identify the subsets of cells within the lymphoid tissue that expressed DRAK2, we examined expression of DRAK2 protein by Western blot. In the thymus, DRAK2 expression is low during the double negative (DN) stage, then is increased during the double positive (DP) stage, and continues to increase in the single positive (SP) stage (Figure 1B). In peripheral lymphoid tissues, DRAK2 expression is highest in B cells but is also expressed at high levels in both CD4⁺ and CD8⁺ T cells. DRAK2 is not expressed at significant levels in NK cells, macrophages, or dendritic cells. Due to the lymphoid-specific nature of DRAK2 and the ability of its family members to induce apoptosis, we examined the function of DRAK2 by generating *Drak2*^{-/-} mice. We note that analysis of the mouse genome revealed that there is no mouse homolog to human DRAK1.

No Defect in Negative Selection or Apoptosis in *Drak2*^{-/-} T Cells

Drak2-deficient mice were generated by homologous recombination in 129SVJ embryonic stem cells targeted with a construct that replaced a 400 bp fragment encom-

passing the intron 2/exon 3 junction and most of exon 3 (which contains the lysine residue required for ATP binding) with a neomycin resistance cassette (Supplemental Figure S1A available online at <http://www.immunity.com/cgi/content/full/21/6/781/DC1/>). *Drak2*^{-/-} mice were recovered at the expected Mendelian frequencies and did not exhibit any gross abnormalities when compared to littermate controls.

Given the reported ability of DRAK2 to induce apoptosis after ectopic expression in cell lines (Matsumoto et al., 2001; Sanjo et al., 1998), we tested whether negative selection in *Drak2*^{-/-} mice was altered in two in vivo models. First, *Drak2*^{-/-} mice were crossed to *H-Y* TCR transgenic mice. This TCR is specific for a peptide from the male antigen bound to H-2D^b, and T cells expressing this receptor are deleted before the DP stage of development in male mice (Kisielow et al., 1988). Second, the role of DRAK2 was evaluated in late-stage negative selection in *AND* TCR transgenic mice that expressed H-2^{b/s} (Page et al., 1998). In this model of negative selection, thymocytes are deleted after the DP stage, but before the CD4⁺CD8⁻ (CD4SP) stage. A deficiency in *Drak2* did not affect the loss of self-reactive T cells in either model (data not shown). In addition, the role of DRAK2 in thymocyte apoptosis was tested in vitro with several apoptosis-inducing agents. Surprisingly, there were no significant differences in thymocyte apoptosis

induced by anti-CD3, peptide-pulsed antigen-presenting cells (APC), anti-Fas, dexamethasone, staurosporine, etoposide, or γ -irradiation (Supplemental Figure S2 and data not shown). Together, these data suggest that DRAK2 does not play an essential role in induction of thymocyte apoptosis or negative selection.

Enhanced Positive Selection in the Absence of DRAK2

Although there was no defect in thymocyte negative selection or differences in total thymic cellularity, *Drak2*^{-/-} thymuses had a slight, yet statistically significant, increase in the percent of CD4SP thymocytes compared to wild-type (wt) mice (Supplemental Figure S3A). This increase coincided with a concomitant decrease in the percent of DP thymocytes and was enhanced when *Drak2*^{-/-} mice were bred to mice expressing MHC class II-restricted TCR transgenes, either *OT-II* or *AND* (Supplemental Figure S3A and data not shown). However, there was no effect on subset distribution when *Drak2*^{-/-} mice were bred to *OT-I* or *P14* mice, MHC class I-restricted TCR transgenic mice (Supplemental Figure S3A). Thus, the absence of *Drak2* enhanced development of CD4⁺ thymocytes but had little effect on CD8⁺ thymocytes. In addition, the levels of CD5 and CD69 were slightly increased on *OT-II;Drak2*^{-/-} and *OT-I;Drak2*^{-/-} DP thymocytes compared to wt DP thymocytes (Supplemental Figure S3B). This increase in CD5 and CD69 expression in the absence of *Drak2* suggests that the intensity of the signal through the TCR was enhanced in *Drak2*^{-/-} thymocytes. We conclude that DRAK2 negatively regulates signals initiated through the TCR.

An Increase in Memory-like Cells in the Absence of DRAK2

An analysis of the lymphocytes present in the spleens of *Drak2*-deficient mice revealed an increase in the number of cells with characteristics of memory T cells. The percent of CD62L^{lo} cells was consistently increased, but the difference in other memory markers, such as CD44 and CD45RB was more variable (Supplemental Figure S4A). There was no difference between *Drak2*^{-/-} and wt T cells in the expression of transient markers of activation such as CD69, CD25, or LY6C (Supplemental Figure S4B). The percent of CD62L^{lo} cells was the largest and most consistent difference between wt and *Drak2*^{-/-} T cells, whereas this increase was not observed in *Drak2*^{-/-} mice expressing the *OT-I* or *OT-II* TCR transgenes (data not shown). This suggests that DRAK2 does not directly affect CD62L expression and that the appearance of these cells may arise as a result of an encounter with environmental or self-antigens. This further suggests that in the absence of *Drak2*, the T cells responded to antigens that are otherwise ignored by the immune system.

Hyperproliferation of *Drak2*^{-/-} T Cells to Suboptimal Antigen Stimulation

If DRAK2 acts as a negative regulator of T cell signaling, then one would predict that *Drak2*^{-/-} T cells would be hypersensitive to antigenic stimulation. To test this, we stimulated *OT-I;Drak2*^{-/-} T cells in vitro with the agonist peptide OVAp or an altered form of this peptide G4. The

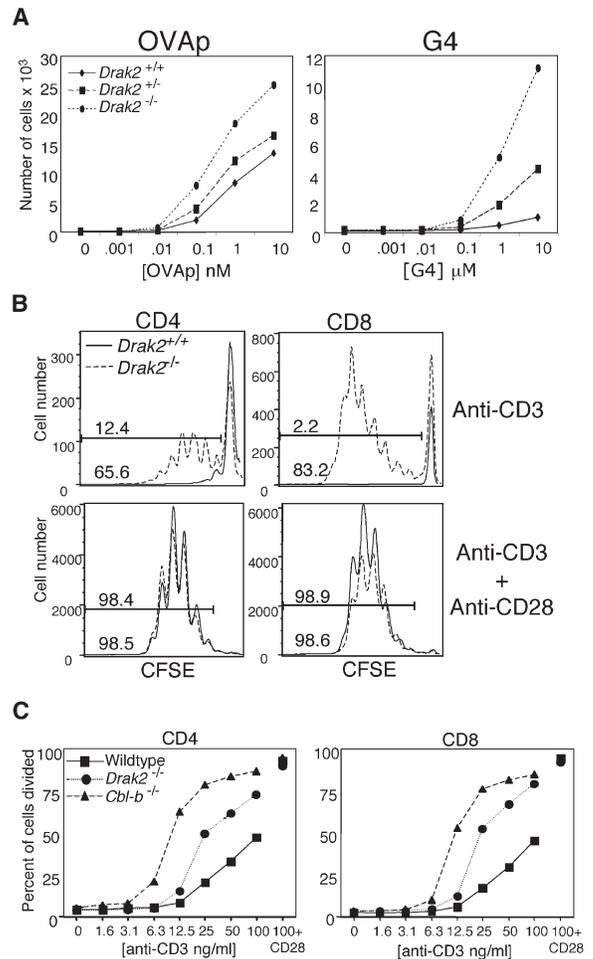


Figure 2. Increased Sensitivity of *Drak2*^{-/-} T Cells to Suboptimal Stimuli

(A) *OT-I;Drak2*^{+/+}, *Drak2*^{-/-}, and *Drak2*^{+/-} splenocytes were labeled with CFSE and incubated with various amounts of the strong agonist peptide, OVAp, or the weak agonist, G4. After 2 days, the number of CFSE^{lo}, CD8⁺ T cells was determined by flow cytometry. These data are representative of three separate experiments.

(B) T cells were purified by negative depletion from the spleen of *Drak2*^{+/+} (solid lines) and *Drak2*^{-/-} (dashed lines) littermates (N3 to C57BL/6), labeled with CFSE, and stimulated with plate bound anti-CD3. The numbers represent the percent of cells that have divided at least once. The percentage for *Drak2*^{+/+} is shown above the line and the percentage for *Drak2*^{-/-} is below the line.

(C) Lymph node T cells from wt, *Drak2*^{-/-}, and *Cbl-b*^{-/-} mice (\geq N6 to C57BL/6) were purified by negative depletion and subsequently separated with CD62L magnetic beads. The CD62L⁺ fraction was CFSE labeled and stimulated with plate bound anti-CD3. The cells were greater than 95% Thy1⁺, CD62L⁺. The data are representative of three experiments.

G4 peptide varies from the agonist peptide by one amino acid residue but has a 8-fold reduced affinity for the *OT-I* TCR and therefore induces a weak T cell response (Rosette et al., 2001). Consistent with a deficiency in a negative regulator, *Drak2*^{-/-} T cells proliferated more in response to antigenic stimulation, and this difference was greater in response to the weak agonist (Figure 2A).

The observed phenotype may be the result of hypersensitive T cells; however, it may also be caused by the

APC because DRAK2 is also expressed in B cells (Figure 1B). To determine whether the effect of *Drak2* ablation was T cell autonomous, the APC were exhaustively depleted by magnetic sorting, and the purified T cells were stimulated in vitro with anti-CD3 alone. Wt T cells were completely unresponsive to low doses of plate bound anti-CD3, while *Drak2*^{-/-} T cells exhibited a robust proliferative response even in the absence of any source of costimulation (Figure 2B). Addition of anti-CD28 antibody evoked a response in *Drak2*^{-/-} and *Drak2*^{+/-} T cells that was virtually indistinguishable (Figure 2B). These data demonstrate that in the absence of *Drak2*, T cells responded to suboptimal stimulation that did not evoke a response in wt T cells, and this effect was independent of the APC population.

Given the fact that there were more CD62L^{lo} cells in the spleen of *Drak2*^{-/-} mice, it was possible that the hypersensitivity of *Drak2*^{-/-} T cells was a consequence of an increase in the number of CD62L^{lo} cells. To address this possibility, the CD62L^{lo} cells were removed from the purified T cells by magnetic sorting, and the remaining T cells were stimulated in vitro with anti-CD3. Even in the absence of CD62L^{lo} cells, *Drak2*^{-/-} T cells proliferated to doses of anti-CD3 that did not induce proliferation of wt T cells, and again both proliferated equally well when costimulation was present (Figure 2C).

Because DRAK2 functions as a negative regulator of T cell activation, we compared the response of *Drak2*^{-/-} T cells to T cells lacking another regulatory molecule, Cbl-b. Cbl-b reduces the amount of signaling through the TCR, and in its absence, T cells are hypersensitive to antigenic stimulation (Bachmaier et al., 2000; Chiang et al., 2000; Naramura et al., 2002). As shown previously, *Cbl-b*^{-/-} T cells were hypersensitive to suboptimal doses of anti-CD3 similar to *Drak2*^{-/-} T cells (Figure 2C). However, the sensitivity of the *Cbl-b*^{-/-} T cells was greater than that of the *Drak2*^{-/-} T cells, suggesting that DRAK2 and Cbl-b may inhibit separate signaling pathways.

***Drak2*^{-/-} T Cells Produce More Cytokines and Express Higher Levels of Activation-Induced Costimulatory Molecules**

In order to understand the mechanism in which DRAK2 regulates T cell activation, we examined other consequences of TCR stimulation such as cytokine production and expression of activation-induced molecules. First, we measured the amount of IL-2 produced by naive T cells in response to anti-CD3 alone. Analogous to the proliferation results, *Drak2*^{-/-} T cells produced more IL-2 than wt T cells, but not as much IL-2 as *Cbl-b*^{-/-} T cells (Figure 3A). This provides at least one explanation for the increased sensitivity of *Drak2*^{-/-} T cells to suboptimal stimuli. We also measured the amount of IL-4 and IFN γ in unpolarized and Th1/Th2 polarized T cells. Both wt and *Drak2*^{-/-} T cells produced the appropriate cytokines under Th1 and Th2 skewing conditions (Figure 3B). In addition, under nonskewing conditions, *Drak2*^{-/-} T cells produced more IFN- γ , IL4, and IL-2 compared to controls.

In addition to cytokine production, we analyzed the expression level of various costimulatory molecules on purified, naive T cells before and after activation. Prior to T cell activation, the levels of the cytokine receptors,

costimulatory molecules, and TNFR family members were equivalent between *Drak2*^{-/-} and wt T cells (Figure 3C). However, after suboptimal stimulation with anti-CD3, the levels of various costimulatory markers varied between wt and *Drak2*^{-/-} T cells. The greatest difference was in the level of CD25 (Figure 3C). The increased expression of CD25 was highest at 24 hr and maintained for at least 72 hr after stimulation (Figure 3C). Likewise, the expression levels of other costimulators including IL-7R, ICOS, CD27, OX40, and 41BB were also increased on *Drak2*^{-/-} T cells in response to suboptimal stimulation (Figure 3C). Consistent with previous experiments, the levels of these costimulatory molecules were similar between wt and *Drak2*^{-/-} T cells when stimulated by anti-CD3 and anti-CD28. Although several positive costimulatory molecules increased in the absence of *Drak2*, there were also increases in two negative regulators, PD-1 and CTLA-4. These results exemplify the robust regulation of the immune response whereby enhanced stimulation is self-limiting.

No Defect in Peripheral T Cell AICD and an Increased Sensitivity to Costimulation

As T cells are stimulated and respond to cytokines, there is ongoing cell death. Although there was no defect in apoptosis of thymocytes, we considered the possibility that the hyperproliferative phenotype of *Drak2*^{-/-} peripheral T cells resulted from diminished cell death after activation. In order to analyze the level of apoptosis associated with T cell activation, purified T cells were labeled with CFSE, stimulated with anti-CD3, and stained with Annexin V to detect the percentage of apoptotic cells at each round of cell division. T cells from wt mice did not undergo proliferation without costimulation (Figure 4A), and two effects became apparent as anti-CD28 was titrated into culture. At low concentrations of anti-CD28, a large proportion of the wt T cells progressed through the cell cycle, but a large percentage of those cells died at each round of division. As costimulation was increased, the same proportion of cells proliferated, but the apoptosis was dramatically reduced. This illustrates the two activities mediated by costimulation, proliferation associated with cytokine production and survival. The question was whether *Drak2*^{-/-} T cells would divide without the concomitant cell death. *Drak2*^{-/-} T cells were capable of dividing in response to anti-CD3 stimulation with minimal anti-CD28 antibody, but the *Drak2*^{-/-} cells still exhibited a proportional cell death (Figure 4A). In fact, the response to anti-CD28 was virtually identical to that elicited in wt cells with the exception that the dose response was shifted to lower concentrations of agonistic anti anti-CD28 antibody.

The net recovery of cells from these experiments was plotted as a function of the time to reflect both proliferation and apoptosis (Figure 4B). Wt CD8⁺ T cells expanded at low concentrations of anti-CD28, whereas wt CD4⁺ T cells required 10- to 100-fold more costimulation to achieve the same cell production. This was similar for *Drak2*^{-/-} T cells with the exception that both of the responses required even lower concentrations of anti-CD28. The loss of *Drak2* would therefore not appear to obviate the need for costimulation, but rather T cells are more sensitive to costimulation as measured by either

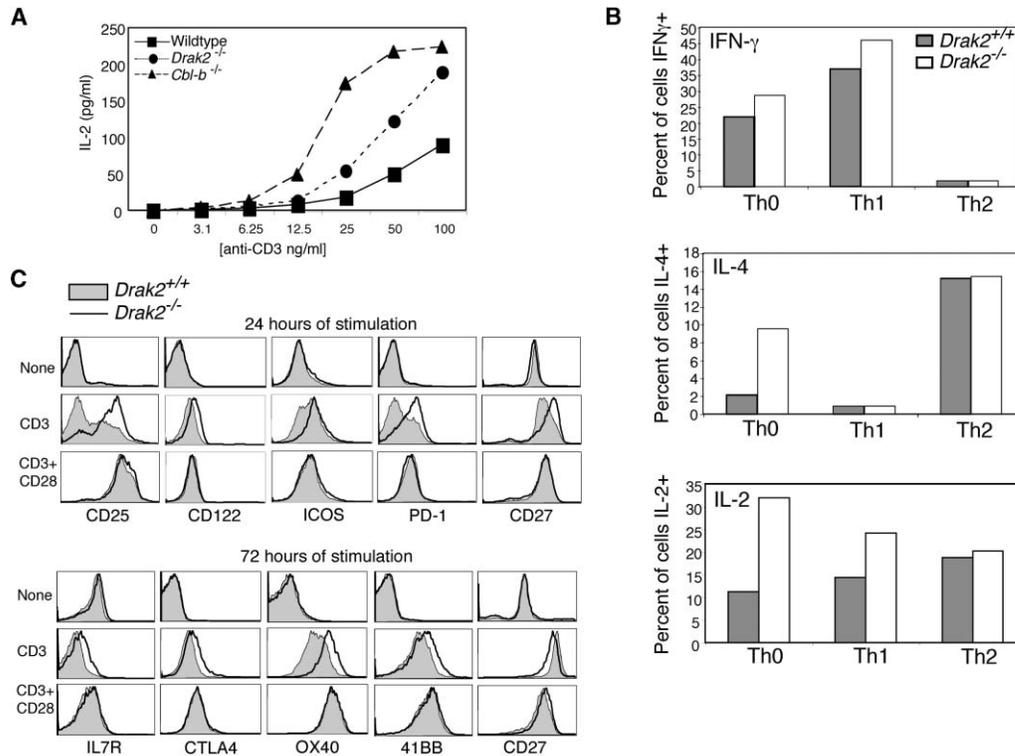


Figure 3. Increased Cytokine Production and Surface Marker Upregulation by *Drak2*^{-/-} T Cells

(A) A sample of the culture supernatant was removed from each well of the experiment described in Figure 2C after 24 hr of stimulation. The amount of IL-2 in each sample was measured by ELISA. This experiment is representative of at least three experiments.

(B) Purified CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of Th1, Th2, or nonskewing conditions for 3 days. The cells were then rested in the presence of IL-2 for 3 days, then restimulated with anti-CD3 in the presence of Brefeldin A for 4 hr and stained for intracellular cytokine and analyzed by flow cytometry. The data are representative of two separate experiments.

(C) Purified T cells were stimulated with 20 ng of plate bound anti-CD3 or 100 ng of plate bound anti-CD3 with soluble anti-CD28 for either 24 or 72 hr, harvested, and stained with antibodies to the various costimulatory molecules. The filled histograms represent electronically gated CD4⁺, *Drak2*^{+/+} T cells and the open represent CD4⁺, *Drak2*^{-/-} T cells. Data are representative of three separate experiments.

proliferation or survival. Together these data demonstrate that the enhanced proliferation exhibited by *Drak2*^{-/-} T cells did not arise from a defect in cell death associated with activation.

The Impact of DRAK2 on an In Vivo Immune Response

Although there was no defect in apoptosis of *Drak2*^{-/-} T cells in vitro, we wanted to confirm that apoptosis after an in vivo immune response was also intact in the absence of *Drak2*. Thus, mice were infected with lymphocytic choriomeningitis virus (LCMV). At various days postinfection, the number of CD8⁺ T cells specific for the gp33 epitope of LCMV and the number of CD4⁺ T cells specific for gp61 epitope were calculated. As shown in Figure 4C, there was a robust response to LCMV in both wt and *Drak2*^{-/-} mice. Furthermore, the absence of *Drak2* did not affect the contraction phase of the response, supporting the in vitro data measuring cell death (Figure 4C). To be sure that there was not an alteration in the migration of T cells to nonlymphoid tissue, we also counted the number of LCMV-specific T cells in the lung, and the response in this organ was similar to that in the spleen (data not shown). The difference between the number of wt and *Drak2*^{-/-} T cells

that responded to LCMV was not dramatic, which would be predicted for a response in which costimulation is likely to be maximal. In addition to counting the number of LCMV-specific T cells, we also measured the amount of IFN-γ in the serum during the response. Interestingly, the level of IFN-γ present in the serum of *Drak2*^{-/-} mice was greater than that of wt mice before the peak of the response and during the contraction phase (Figure 4D). This is consistent with the fact that *Drak2*^{-/-} T cells make more IL-2, IL-4, and IFN-γ than wt T cells in vitro (Figure 3B), suggesting that *Drak2*^{-/-} T cells retain some aspects of hypersensitivity in vivo.

Subcellular Localization of DRAK2 and Increased TCR Signaling in Its Absence

In order to understand how DRAK2 influences T cell activation, we looked for differences in signaling molecules downstream of TCR activation. To narrow down the number of possible candidates, we first established whether DRAK2 was localized in the cytoplasm or the nucleus of naive T cells and if this localization changed after activation. Cytoplasmic and nuclear fractions were prepared from purified, wt T cells that were stimulated with anti-CD3 and anti-CD28. In naive, unstimulated T cells, DRAK2 was located in both the cytoplasm and

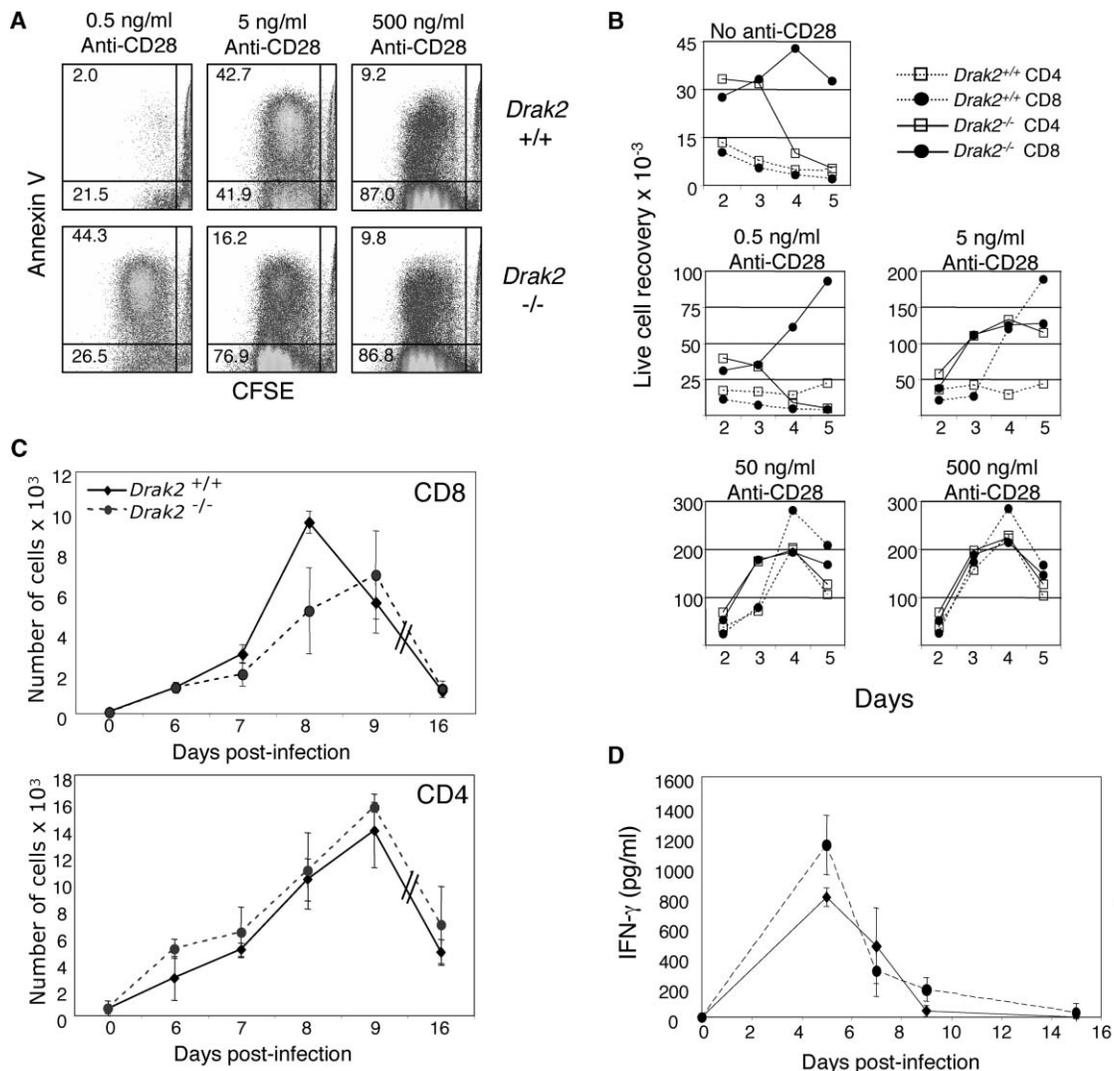


Figure 4. Normal Apoptosis in *Drak2*^{-/-} Peripheral T Cells

(A) Purified T cells were labeled with CFSE and stimulated with plate-bound anti-CD3 in the absence or presence of varying amounts of anti-CD28. After 4 days, cells were harvested and labeled with antibodies against CD4 and CD8, and stained with Annexin V. Data for CD4⁺ cells from *Drak2*^{+/+} and *Drak2*^{-/-} littermates (N3 to C57BL/6J) are shown. The numbers represent the percentage of cells in each quadrant.

(B) The number of live cells recovered from these cultures is shown in the graphs. The solid lines represent *Drak2*^{-/-} T cells, and the dashed lines represent *Drak2*^{+/+} T cells. The CD4⁺ cells are denoted by the open square (\square), and the CD8⁺ by the closed circle (\bullet).

(C) *Drak2*^{+/+} and *Drak2*^{-/-} littermates (>N6 to C57BL/6J) were infected with 2×10^5 PFU of LCMV. T cells were isolated from the spleen and stimulated in vitro for 5 hr in the presence of gp33 or gp61 and Brefeldin A, and stained for intracellular IFN- γ to detect LCMV-specific T cells. The number of CD8⁺, CD44⁺, IFN- γ ⁺ (top) and CD4⁺, CD44⁺, IFN- γ ⁺ (bottom) cells is plotted. Each point is the average of three mice. The data are representative of four separate infections.

(D) Serum was isolated from the blood of LCMV-infected mice at each time point, and the amount of IFN- γ present was measured by ELISA. These data are representative of three experiments.

nucleus (Figure 5A). Interestingly, 2 min after activation, the level of DRAK2 in the nucleus decreased and remained low for up to 48 hr (Figure 5A). The level of DRAK2 in the cytoplasm also decreased after activation, but this was not evident until 24 hr after stimulation (Figure 5A). At this time, the level of *Drak2* mRNA was also lower than naive T cells (Figure 5B). These experiments demonstrate that DRAK2 could interact with both cytoplasmic and nuclear proteins and that TCR stimulation directly affects DRAK2 localization. Furthermore, this expression pattern is similar to other negative regu-

lators of T cell activation such as Foxj1, Tob, and LKLF (Kuo et al., 1997; Lin et al., 2004; Tzachanis et al., 2001).

Next, we tested whether well-characterized signaling pathways downstream of the TCR were altered in the absence of *Drak2*. First, we measured calcium flux in response to TCR stimulation. In *Drak2*^{-/-} CD4⁺ T cells, the amount of calcium fluxed in response to anti-CD3 stimulation was increased relative to wt T cells (Figure 5C); however, there was no difference when the T cells were stimulated with ionomycin, which bypasses the signal through the TCR. This provides evidence that the

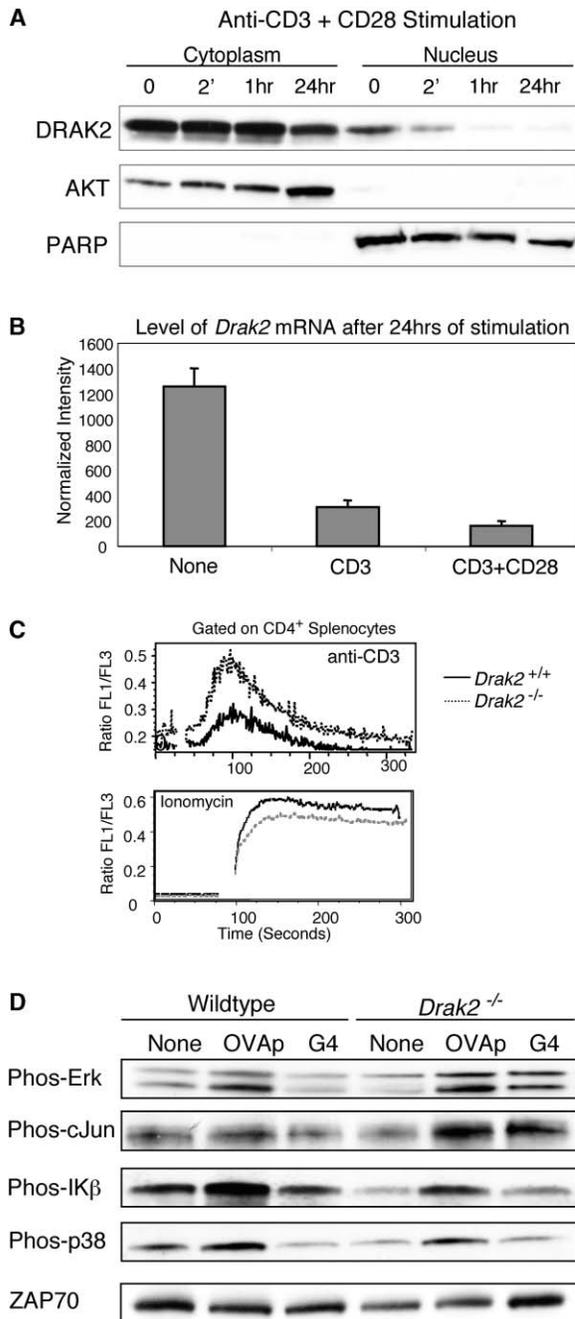


Figure 5. Cellular Localization of DRAK2 and Increased Proximal TCR Signaling in *Drak2*^{-/-} T Cells

(A) T cells were purified from the lymph nodes of wt mice and stimulated with anti-CD3 and anti-CD28 for either 2 min, 1 hr, or 24 hr. The cells were then lysed and separated into cytoplasmic and nuclear fractions and analyzed by Western for DRAK2 expression. To confirm that each fraction was pure, the blot was stripped and reprobed with anti-AKT (cytoplasmic) and anti-PARP (nuclear). These data are representative of two separate experiments.

(B) T cells were purified from the lymph nodes of wt mice and stimulated with anti-CD3 or anti-CD3 and anti-CD28 for 24 hr. RNA was isolated and hybridized to Affymetrix GeneChip arrays. Each sample was done in duplicate, and the average of each normalized intensity is shown.

(C) T cells from *Drak2*^{+/+} (solid lines) and *Drak2*^{-/-} (dashed lines) mice were purified and labeled with Fluo-4 and Fura red, stained

absence of *Drak2* affects early events in TCR signaling. To test if other signaling molecules were altered, we stimulated *OT-I;Drak2*^{-/-} and *Drak2*^{+/+} T cells for 5 min with peptide-pulsed APC. In these experiments, the APC were pulsed with the agonist peptide OVAp or the suboptimal peptide G4. Consistent with an increase in calcium flux, there was also an increase in the phosphorylation of ERK1/2 and c-Jun in *Drak2*^{-/-} T cells compared to wt cells (Figure 5D). Like the proliferation data, the difference was greater in response to the suboptimal stimulus. Interestingly, not all TCR signaling pathways were affected as there was no difference in phosphorylation of JNK, p38, or I κ B- α , a molecule in the NF- κ B pathway. In addition, we did not observe a difference in the phosphorylation of molecules proximal to the TCR including TCR ζ , LCK, and ZAP70 (data not shown). Although we have not identified a molecule with which DRAK2 directly interacts, in its absence, critical pathways downstream of TCR signaling are overstimulated.

Drak2^{-/-} Mice Are Resistant to EAE

The data presented thus far establish that DRAK2 is a negative regulator of T cell activation, and in its absence, T cells are hypersensitive to suboptimal stimuli. Based on other examples of T cell hypersensitivity, we predicted that *Drak2*^{-/-} mice would be more susceptible to autoimmune disease. To test for spontaneous autoimmunity, we aged 9 *Drak2*^{+/+} and 15 *Drak2*^{-/-} littermates for at least 15 months and analyzed the major organs of these mice for cellular infiltrate. In addition, we measured the amount of anti-nuclear and anti-dsDNA antibodies in the serum of the aged mice. Of the 15 *Drak2*^{-/-} mice, only one mouse had a very mild infiltrate in the pancreas. In the remaining wt and *Drak2*^{-/-} mice, there were no signs of cellular infiltration (data not shown). In addition, none of the *Drak2*^{-/-} mice had higher levels of autoantibodies than the wt littermates (data not shown). Contrary to expectations, there was no obvious increase in these indicators of autoimmune disease.

In order to test if *Drak2* had an impact on induced autoimmune disease, we compared the responses of wt, *Drak2*^{-/-}, and *Cbl-b*^{-/-} mice in EAE, a model of multiple sclerosis. In this model, C57BL/6 mice are immunized with a peptide from myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant. Induction of disease is dependent on MOG-reactive CD4⁺ T cells that cross the blood-brain barrier and induce inflammation in the spinal cord and the brain. The onset of disease in wt mice occurred close to day 14 after the initial immunization and reached a maximum around

with antibodies against CD4 and CD8, then stimulated with biotin anti-CD3 and streptavidin. Calcium flux was measured by taking a ratio of the change in fluorescence of the two dyes as measured by flow cytometry. Data represent the calcium flux from CD4⁺ cells. (D) Purified *OT-I;Drak2*^{+/+} and *OT-I;Drak2*^{-/-} CD8⁺ T cells were stimulated with peptide-pulsed, magnetically labeled APC for 5 min at 37°C. Each sample was then run over a magnet to separate the APC and T cells. The T cell fraction was lysed and analyzed by Western blot analysis. The amount of ZAP70 is displayed to show that lysates from an equal number of T cells were analyzed. The data represent three separate experiments.

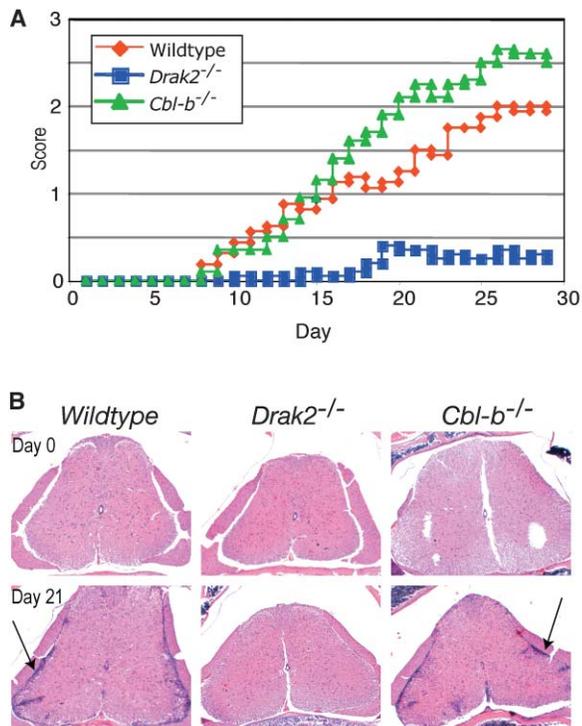


Figure 6. *Drak2*^{-/-} Mice Are Resistant to EAE

(A) EAE was induced with immunization in the hind flanks with a MOG peptide emulsified in complete Freund's adjuvant. The mice were monitored daily for signs of disease and given a score according to the severity of disease: 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; 4, complete hind limb paralysis and partial forelimb paralysis, and/or moribund. The experiment was performed two times, and the data were pooled together. Each line represents the average score for ten *Cbl-b*^{-/-}, ten *Drak2*^{-/-}, eight wt mice. The *Cbl-b*^{-/-} mice were N12 to C57BL/6J and the *Drak2*^{-/-} mice were at least N6 to C57BL/6, and littermates of the *Drak2*^{-/-} mice were the wt mice.

(B) Spinal cords were removed from mice 21 days after EAE immunization, fixed, decalcified, and stained with Hematoxylin and Eosin. The images are 40× magnification, and the arrows indicate an example of mononuclear infiltrate.

day 26 (Figure 6A and Table 1). As previously reported (Chiang et al., 2000), *Cbl-b*^{-/-} mice were more susceptible to EAE in that a greater number of mice exhibited signs of disease, and the severity of this disease was elevated compared to wt mice (Table 1). Contrary to our expectations, *Drak2*^{-/-} mice were not more susceptible to EAE, but rather they were extremely resistant to disease (Figure 6A). Both the incidence and the severity

Table 1. The Incidence and Severity of EAE

	Incidence	Mean Onset	Mean Severity
Wt	6/8 (75%)	14.4	2.3
<i>Drak2</i> ^{-/-}	2/10 (20%)	18.3	0.7
<i>Cbl-b</i> ^{-/-}	8/10 (80%)	15.6	2.8

The incidence of disease reflects the number of mice that scored a two or higher. The mean onset is the average of the day when each mouse first scored one. The mean severity is the average of the highest score reached by each mouse.

of disease were dramatically reduced in *Drak2*^{-/-} mice (Table 1). In accord with symptoms, *Drak2*^{-/-} mice exhibited a reduced number of infiltrating cells in the nervous system (Figure 6B), although there were similar numbers of MOG-specific cells in the spleen and the draining lymph nodes 20 days after immunization (data not shown). Therefore, both *Drak2*^{-/-} and *Cbl-b*^{-/-} T cells were hypersensitive to suboptimal stimuli; however, the impact this hypersensitivity had on autoimmunity was completely contradictory. In fact, thus far, a deficiency in *Drak2* is the only model of T cell hypersensitivity that results in a resistance to autoimmunity.

Discussion

DRAK2 is unique in that it is one of the few proteins that is specifically expressed in lymphoid tissue. Based on its similarity to DAP-like family members and direct experimentation (Sanjo et al., 1998), we predicted that DRAK2 would play a role in T cell apoptosis. However, we demonstrate here that the absence of *Drak2* does not affect apoptosis of T cells in a number of different assays. This includes apoptosis of thymocytes in vitro and two models of in vivo negative selection. In addition, there was no defect in death of mature T cells after in vitro stimulation or after an in vivo immune response. Furthermore, overexpression of *Drak2* in Jurkat T cells did not result in an increase of apoptosis (data not shown). Therefore, we conclude that DRAK2 does not play a critical role in T cell apoptosis.

An analysis of *Drak2*^{-/-} mice revealed that it encodes a negative regulator of T cell activation, and this is supported by several distinct phenotypic differences in T cell development and activation. First, positive selection of CD4⁺ T cells was enhanced as illustrated by the increase in the number of CD4⁺ thymocytes and an increase in the levels of CD5 and CD69 expression. There was an increase in memory-like T cells that was antigen specific. The requirement for costimulation was dramatically reduced although *Drak2*^{-/-} T cells retained sensitivity to costimulation (Figure 2B). In fact, the effects of costimulation, including both proliferation and survival, were identical in *Drak2*^{-/-} and wt T cells, with the exception that *Drak2*^{-/-} T cells responded to lower doses of anti-CD28 antibodies (Figures 4A and 4B). Control experiments showed this hypersensitivity was not due to differences in APC or due to the fact that there were more CD62L^{lo} T cells in *Drak2*^{-/-} mice. Together, these data reveal that DRAK2 functions to negatively regulate signals involved in T cell activation.

The most surprising characteristic observed in *Drak2*^{-/-} mice was the resistance to EAE despite the T cell hypersensitivity. In other models of negative T cell regulation that have been tested for autoimmunity, the mice were either equally susceptible or more susceptible to disease when compared with wt mice. In fact, deficiencies in *Cbl-b* (Bachmaier et al., 2000; Chiang et al., 2000), *Itch* (Fang et al., 2002), *Sts-1* and *Sts-2* (Carpino et al., 2004), *Mgat5* (Demetriou et al., 2001), *SHP-1* (Kozlowski et al., 1993; Shultz et al., 1993; Tsui et al., 1993), *Foxj1* (Lin et al., 2004), *CTLA-4* (Tivol et al., 1995; Waterhouse et al., 1995), *PD-1* (Nishimura et al., 1999), *SHIP* (Helgason et al., 1998), and *Pten* (Suzuki et al.,

2001) all resulted in an increased susceptibility to autoimmunity. Thus far, DRAK2 appears to be the only negative regulatory molecule that nonetheless conveys resistance to autoimmunity.

Due to the complexity of EAE, there are many opportunities for a regulatory molecule to affect disease. It is possible that there is a defect in CD4⁺ T cell expansion and survival in response to MOG immunization; however, this does not seem likely given the results of experiments measuring the T cell response in culture or in response to an infectious agent *in vivo*. In addition, at day 20 after immunization with MOG, there were similar numbers of antigen-reactive cells in the spleen and draining lymph nodes of *Drak2*^{-/-} and wt mice (data not shown). Rather, the loss of disease appears to originate from a loss of CNS infiltration. The number of MOG-specific cells in the spinal cord and brain of the *Drak2*^{-/-} mice was reduced compared to wt and *Cbl-b*^{-/-} mice, and this number correlated strongly with disease. We are currently investigating the possible reasons for the difference in migration to the central nervous system (CNS), and obvious possibilities include a dysregulation of adhesion molecules, chemokines, or chemokine receptors. Consistent with this possibility, there were more CD62L^{lo} cells in the spleen of *Drak2*^{-/-} mice compared to wt mice (Supplemental Figure S4).

Another way in which the absence of *Drak2* could affect autoimmunity is by altering the cytokine expression of T cells. The amount and timing of cytokine expression is critical in EAE. An increase in Th2 cytokines can impart resistance to EAE and, paradoxically, IFN- γ has also been suggested to play an inhibitory role (Pedotti et al., 2003; Willenborg et al., 1996). In addition, T cells deficient in MAP kinase phosphatase 5 (*Mkp5*) have elevated levels of activated JNK and produce more cytokines than wt T cells; however, *Mkp5*-deficient mice are resistant to EAE (Zhang et al., 2004). Clearly, the role of cytokines in EAE is not completely understood, but it is conceivable that an increase in either IFN- γ or IL-4 by *Drak2*^{-/-} T cells could result in decreased EAE (Figure 3B).

Drak2-deficiency could affect EAE as a consequence of altered B cell regulation. Previous experiments showed that B cells can play a protective role in EAE through the production of IL-10 (Cross et al., 2001; Dittel et al., 2000; Wolf et al., 1996). Because DRAK2 is highly expressed in B cells, it is possible that *Drak2*^{-/-} B cells are also hypersensitive and produce more disease-inhibiting IL-10. Consistent with this, *Drak2*^{-/-} B cells were hyperproliferative to suboptimal stimuli *in vitro* (data not shown).

Finally, the effect of *Drak2* on autoimmunity may be through regulatory T cells. Given that *Drak2*^{-/-} conventional T cells are hypersensitive to antigenic stimulation, it is possible that *Drak2*^{-/-} regulatory T cells are also hypersensitive to stimulation, and the response of the regulatory T cells could dominate over that of the conventional CD4⁺ T cells that induce disease. However, *in vitro*, *Drak2*^{-/-} regulatory T cells did not suppress T cell activation more effectively than wt T cells (data not shown). In addition, we did not detect an increase in the number of CD4⁺CD25⁺CD69⁻ or CD4⁺GITR⁺T cells in the CNS or draining lymph nodes at day 20 after MOG immunization in *Drak2*^{-/-} mice compared to wt

mice (data not shown). However, this does not eliminate the possibility that there are more regulatory T cells present early in the response, and this is sufficient to inhibit disease.

In conclusion, the absence of *Drak2* does not appear to negatively impact the health or fecundity of mice. Yet, a *Drak2* deletion renders mice resistant to autoimmune disease though fully capable of mounting a normal immune response to an infectious virus. It thus presents an evolutionary enigma—what was the selective pressure to evolve a regulatory molecule that inhibits the effectiveness of an immune response and increases the propensity for autoimmunity? Whatever the answer, we note that DRAK2 constitutes a cell type-specific target that could be inhibited to treat autoimmune disease without the risks normally associated with immune inhibition.

Experimental Procedures

Mice

Drak2^{-/-} mice were generated by homologous recombination in 129SVJ embryonic stem cells. The genomic clone corresponding to *Drak2* was obtained by screening a 129SVJ mouse genomic lambda phage library (Stratagene, La Jolla, CA) by using full-length *Drak2* cDNA as a probe. Most *Drak2*^{-/-} mice analyzed were backcrossed to C57BL/6 at least six generations, unless indicated in the figure legend. In all cases, littermates were used as controls. *Cbl-b*^{-/-} mice were a generous gift from Hua Gu and were backcrossed 12 generations to C57BL/6.

Purification of Lymphoid Populations

DN, CD8⁺, CD4⁺, and postselection DP thymic subsets were purified from C57BL/6 thymuses by sorting with a FACSVantage (Becton Dickinson, San Jose, CA) after magnetic depletion with MACS beads (Miltenyi Biotech, Auburn, CA). Preselection DP thymocytes were obtained from a MHC I/II^{-/-} (β_2M ^{-/-}; I-A^b^{-/-}) thymus and sorted for expression of CD4 and CD8 with the FACSVantage. Peripheral T cells were purified from the lymph nodes by negative selection with biotin-CD4 or CD8 and B220, CD11b, DX5, and MHC class II (I-A^b) antibodies (eBioscience, San Diego, CA), followed by separation with streptavidin-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA). B cells were purified from the spleen by positive selection with B220-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA). NK cells and macrophages were obtained from splenocytes that were first depleted of T and B cells followed by sorting based on CD11b and NK1.1 expression. Macrophages were CD11b⁺ NK1.1⁻ and NK cells were NK1.1⁺ with about half also expressing CD11b. Dendritic cells were obtained from collagenase-digested spleens that were then spun on a BSA gradient. CD19⁺ and Thy1⁺ cells were removed by magnetic sorting, and the remaining cells were sorted for expression of CD11c with a FACSVantage. All subsets were greater than 94% pure.

Flow Cytometric Analyses

Single cells suspensions of thymus, lymph node, and spleen were stained with FITC-, PE-, TC-, PerCP-, and APC-conjugated antibodies against CD4, CD8, CD3, CD5, CD25, CD69, CD44, TCR β , CD62L, PD-1, ICOS, 41BB, CD27, CD127 (IL-7R) Ly6C, CTLA4, and OX40 (Pharmingen, San Diego, CA; Beckman Coulter, Fullerton, CA; and eBioscience, San Diego, CA). Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, San Jose, CA). Analysis was performed with FlowJo software (TreeStar, Inc., Ashland, OR).

CFSE Proliferation

Purified T cells or whole spleen were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene OR) at 2.5 μ M in PBS for 10 min at 37°C, washed with RPMI containing 10% FCS, and incubated with plate bound anti-CD3 or peptide as described below.

CD3 Stimulation

Anti-CD3 was plate bound by first binding goat anti-hamster (Vector Laboratories, Burlingame, CA) followed by the appropriate concentration of anti-CD3 ascites (145-2C11). Anti-CD28 ascites (N37) were added at varying concentrations. After 24–72 hr, T cells were harvested and stained with Annexin V (Pharmingen, San Diego, CA).

Th1/Th2 Skewing

CD4⁺ T cells, purified as described above, were stimulated in Th1 or Th2 conditions as described previously (Rengarajan et al., 2002). After 4 days, the cells were restimulated for 5 hr in the presence of Brefeldin A, fixed, and permeabilized with Cytofix/Cytoperm Plus staining kit (Pharmingen, San Diego, CA). The treated cells were then stained with PE-anti-IL-2, PE-anti-IFN- γ , or PE-anti-IL4 (eBioscience, San Diego, CA).

LCMV Infection

LCMV virus was a generous gift from Raymond Welsh. Mice were infected with 2×10^5 PFU of LCMV via intraperitoneal injection. T cells were isolated from the spleen at various days postinfection and stimulated in vitro for 5 hr in the presence of gp33 or gp61 peptides, IL-2, and Brefeldin A, then stained for intracellular IFN- γ with the cytofix/cytoperm plus kit to detect LCMV-specific T cells. In addition, blood was collected via cardiac puncture in the presence of 15 μ l of 0.5 M EDTA. The blood was spun down and plasma was frozen at -80°C and used to determine levels of IFN- γ by ELISA.

DRAK2 Localization

T cells were purified as described above and stimulated with anti-CD3 and anti-CD28. After the various time points, the cells were harvested into ice-cold PBS to stop the reaction. The cells were lysed in a way to separate the cytoplasmic fraction from the nuclear fraction as described previously (Frasca et al., 2002).

DRAK2 mRNA Expression

Purified T cells were stimulated with plate-bound anti-CD3 for 24 hr, harvested, washed with PBS, and frozen at -20°C . RNA was isolated from frozen cell pellets with RNeasy mini kits (Qiagen, Valencia, CA) and labeled and hybridized as described (Wodicka et al., 1997) to mouse GeneChip arrays (Affymetrix, Santa Clara, CA). Duplicate chips were used for each condition. Data was analyzed with Resolver Gene Expression Data Analysis software (Rosetta Inpharmatics, Seattle, WA).

Calcium Flux

Calcium flux was measured by labeling cells with 2 μ M Fura red, 1 μ M Fluo-4, and 0.2% Pluronic (Molecular Probes, Eugene, OR) for 30 min at room temperature in serum-free media. Cells were washed twice and rested for 20 min in the dark. For stimulation, biotinylated anti-CD3 (eBioscience, San Diego, CA) was added to labeled cells on ice for 15 min, washed, then resuspended with prewarmed streptavidin, and analyzed by flow cytometry. Calcium mobilization was determined by measuring the ratio of Fluo-4:Fura red with FlowJo software (Treestar, Inc., Ashland, OR).

TCR Signaling Analysis

The APC were peritoneal macrophages that were harvested by peritoneal lavage 5 days after injection with 1 ml thioglycollate. The macrophages were labeled with CD11b-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA) and positively selected on a magnetic column. The magnetically labeled APC were then incubated with 5 μ M OVAp or 50 μ M G4 peptides for 3 hr at 37°C . Purified CD8⁺ T cells were added to the APC, spun at high speed for 5 sec, and incubated for an additional 5 min at 37°C . Ice-cold PBS was added to stop the stimulation, and tubes were incubated on ice and centrifuged at 4°C . Each sample was then run over the magnet again to separate the APC and T cells. The T cell fraction was washed in PBS and lysed with the cytoplasmic lysis buffer described above. Equal amounts of total protein were loaded on a 10% SDS gel for Western blot analysis with antiphosphorylated ERK (Rabbit polyclonal), phosphorylated p38 (28B10), phosphorylated I κ B- α (Ser 32, rabbit polyclonal) all purchased from Cell signaling, (Beverly, MA),

ZAP-70 (Transduction Labs, Lexington, KY), and phosphorylated c-Jun (KM-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

EAE

Mice were immunized with 125 μ g of MOG35-55 (MEVGWYRSPFSR VVHLYRNGK, Genemed Synthesis, San Francisco, CA) emulsified in complete Freund's adjuvant containing 0.4 mg of H37Ra mycobacterium tuberculosis (Fisher Scientific, Tustin, CA) in each hind flank. The mice also received 200 ng of Bordetella pertussis toxin (List Biological, Campbell, CA) intraperitoneally immediately after immunization and again on day 2. On day 7, the mice were boosted with another 125 μ g of MOG35-55 emulsified in complete Freund's adjuvant, followed by one injection of 200 ng Bordetella pertussis toxin. Each day, the mice were scored for disease using the following scale: 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; 4, complete hind limb paralysis and partial forelimb paralysis. Mouse cages were coded and individual mice were scored without reference to genotype. Mice were euthanized when they reached a score of 4.

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