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Drak2 Regulates the Survival of Activated T Cells and Is Required for Organ-Specific Autoimmune Disease

Maureen A. McGargill, Carmen Choy, Ben G. Wen, and Stephen M. Hedrick

Drak2 is a serine/threonine kinase expressed in T and B cells. The absence of Drak2 renders T cells hypersensitive to suboptimal stimulation, yet Drak2−/− mice are enigmatically resistant to experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. We show in this study that Drak2−/− mice were also completely resistant to type 1 diabetes when bred to the NOD strain of mice that spontaneously develop autoimmune diabetes. However, there was not a generalized suppression of the immune system, because Drak2−/− mice remained susceptible to other models of autoimmunity. Adoptive transfer experiments revealed that resistance to disease was intrinsic to the T cells and was due to a loss of T cell survival under conditions of chronic autoimmune stimulation. Importantly, the absence of Drak2 did not alter the survival of naive T cells, memory T cells, or T cells responding to an acute viral infection. These experiments reveal a distinction between the immune response to persistent self-encoded molecules and transiently present infectious agents. We present a model whereby T cell survival depends on a balance of TCR and costimulatory signals to explain how the absence of Drak2 affects autoimmune disease without generalized suppression of the immune system. The Journal of Immunology, 2008, 181: 7593–7605.

T cells possess the means to kill infected cells, and if not properly regulated, they can also damage normal, healthy cells, resulting in autoimmune disorders. There are several mechanisms of tolerance in place to prevent such destruction, and one major mechanism is blocking the inappropriate activation of T cells. A number of signaling pathways have been shown to regulate the activation of T cells, and the importance of these molecules is highlighted by the fact that in their absence, mice are more susceptible to autoimmune disease (1).

Drak2 is one such molecule that negatively regulates T cell activation (2). It is a serine/threonine kinase that belongs to the death-associated protein kinase (DAPK) family and is expressed highest in T and B cells (3). All members of the DAPK family have been shown to induce apoptosis upon ectopic expression in various cell types (4). However, the role of Drak2 in apoptosis has been controversial, and its ability to induce apoptosis seems to depend on the level of expression, the cell type studied, and the intracellular localization of Drak2 (4, 5). In T cells, retroviral-directed expression of Drak2 did not result in an increase of cell death (6), whereas transgenic expression of Drak2 in mouse T cells caused an increase in death in the presence of exogenously added IL-2 (7).

Further confounding the issue, extensive studies with Drak2−/− mice revealed that Drak2 is not required for the physiological or induced apoptosis of thymocytes or mature T cells (2). Rather, it is an important negative regulator of lymphocyte activation. Compared with wild-type T cells, Drak2−/− T cells proliferated in response to lower amounts of Ag and were able to proliferate with a reduced dependence on CD28-mediated costimulation. Moreover, Drak2−/− T cells produced more cytokines and expressed higher levels of activation markers than wild-type T cells. In addition, Drak2−/− B cells were hypersensitive to suboptimal stimulation compared with wild-type B cells.

Given these observations, we predicted that Drak2−/− mice would have an increased propensity to develop autoimmune disease, similar to mice that lack other negative regulators of T cell activation (8–21). Instead, we found that Drak2−/− mice were resistant to disease in a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (2). The number of mice that displayed signs of disease and the severity of symptoms were dramatically reduced in Drak2−/− mice. This did not reflect a generalized lack of immune competence, because Drak2−/− mice mounted normal immune responses to acute infection by foreign pathogens, including the following: lymphocytic choriomeningitis virus (LCMV), Listeria monocytogenes (data not shown), and mouse hepatitis virus (MHV) (22). This observation raises the intriguing question of how an increased sensitivity in T cells results in decreased autoimmune disease.

In this study, we demonstrate that the resistance to EAE in Drak2−/− mice is a more general effect and includes resistance to type I diabetes associated with NOD mice. Yet, it does not influence autoimmune diseases that occur as a result of a deficiency in regulatory T cells or diseases in which the pathogenesis is mediated by autoantibodies or cells of the innate immune system. The resistance is T cell intrinsic and originates from an inherent sensitivity to cell death, especially under conditions of strong and chronic Ag-mediated activation. These data reveal that, contrary to...
other members of the DAPK family, Drak2 plays a role in T cell survival, and the phenotypic consequences highlight differences between immune responses to acute infection and chronic reactivity to persistent self-Ags. As such, Drak2 may constitute an effective target for therapeutic intervention of autoimmune diseases without generally compromising immunity.

Materials and Methods

Mice

Drak2<sup>−/−</sup> mice were described previously and backcrossed 13 generations to C57BL/6. Cbl-b<sup>−/−</sup> mice were a gift from H. Gu (Columbia University, New York, NY), and were backcrossed 12 generations to C57BL/6. OT-I mice were a gift from K. Hoggquist (University of Minnesota, Twin Cities, Minneapolis, MN). B6.NZM<sup>−/−</sup> mice were a gift from W. Wakeland (Stanford University School of Medicine, Stanford, CA). TCRα<sup>−/−</sup>, NOD mice, and RAG1<sup>−/−</sup> mice were purchased from Jackson Laboratories. The use of animals in this study was approved by the Institutional Animal Care and Use Committee.

Experimental autoimmune encephalomyelitis

EAE was induced, as described previously (2), and given a score to indicate disease severity, as follows: 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. Mice were euthanized when they reached a score of 4.

For the adoptive transfer EAE, T cells were purified by negative depletion with magnetic beads (Miltenyi Biotec). A total of 8 x 10<sup>6</sup> purified T cells was injected i.v. into TCRα<sup>−/−</sup> mice. After 10–14 days, the mice were bled to confirm similar reconstitution efficiency. One to seven days later, the mice were immunized with myelin oligodendrocyte glycoprotein (MOG) in CFA and scored for disease.

Diabetes in NOD mice

Drak2<sup>−/−</sup> mice were crossed to the NOD strain and screened by PCR for microsatellite markers that identify the 20 NOD susceptibility loci. The sequences for the primers were obtained from the Mouse Genome Informatics (http://www.informatics.jax.org). After four generations of back-crossing, we obtained a Drak2<sup>−/−</sup> male and female that contained all 20 susceptibility loci from the NOD background. These mice were intercrossed, and the offspring were monitored weekly for diabetes. Beginning at 10 wk of age, a drop of blood from the tail vein of female mice was collected and the offspring was monitored weekly for diabetes. Beginning at 10 wk of age, a drop of blood from the tail vein of female mice was collected and the offspring was monitored weekly for diabetes.

Identification of MOG-specific T cells

Cells were harvested from the spleen, blood, or inguinal lymph nodes on various days following immunization. A total of 1 x 10<sup>6</sup> cells was stimulated in vitro with 30 μg of MOG<sub>35–55</sub> peptide (Genemed Synthesis) for 2 h. Monensin was added to the cultures at a final concentration of 1 μg/ml, and the cells were cultured for an additional 2.5 h. The cells were stained with Abs to CD4, CD8, and CD44; fixed and permeabilized with the BD Cytofix/Cytoperm Solution Kit (BD Biosciences); and stained with an Ab to detect intracellular IFN-γ.

Proliferation assay

Cells were harvested from the spleen 15 days following immunization with MOG and CFA. A total of 2 x 10<sup>6</sup> cells was plated in triplicate in a 96-well plate with decreasing amounts of MOG<sub>35–55</sub> peptide and incubated at 37°C for 2 days. [3H]Thymidine was added for the last 8 h of culture, and the amount of incorporated [3H]thymidine was determined.

ELISA

Cells were harvested from the spleen, blood, or inguinal lymph nodes various days following immunization. A total of 1 x 10<sup>6</sup> cells was plated in a 96-well plate with 30 μg of MOG<sub>35–55</sub> peptide and cultured for 24 h. The cells were centrifuged, and the supernatant was removed, frozen, and analyzed for the presence of the indicated cytokines with a Beadlyte Mouse MultiCytokine Flex Kit (Millipore).

To measure autoantibodies, plasma was collected from each mouse and analyzed with anti-dsDNA Ab and anti-nuclear Ab ELISA kits (Alpha Diagnostic).

Regulatory T cell assay

Naive and regulatory T cells were purified from splenocytes with a CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cell Isolation Kit (Miltenyi Biotec). A total of 5 x 10<sup>5</sup> naive T cells was stimulated with 5 x 10<sup>4</sup> mitomycin C-treated spleenocytes and 1 μg/ml soluble anti-CD3 (2C11). Decreasing amounts of regulatory T cells were added to the culture and incubated at 37°C for 3 days. [3H]Thymidine was added for the last 8 h of culture.

Flow cytometry

To detect Foxp3<sup>+</sup> T cells, suspensions were stained with Abs to CD4, CD8, and CD25 (eBioscience), and then fixed, permeabilized, and stained with an Ab to Foxp3 with the Mouse Regulatory T Cell Staining Kit (eBioscience). Annexin V binding was measured by staining the cells with Abs to Thy1, CD4, and CD8 (eBioscience), followed by two washes with PBS, and incubated with 1 μl of annexin V-PE (Invitrogen) in 100 μl of annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) for 15 min at room temperature. A total of 150 μl of annexin V binding buffer was added to each sample and analyzed with a FACSCalibur (BD Biosciences).

Isolation of lymphocytes from the brain

Brains were removed from mice on various days following immunization with MOG and CFA, and cell suspensions were made with a metal screen. The cell suspension was centrifuged, resuspended in 40% Percoll (Amer sham), underlayered with 67% Percoll, and centrifuged at 2500 rpm for 20 min with no brake. The interface was removed, transferred to a new tube, washed with medium, and stained with Abs and annexin V for analysis by flow cytometry.

Stimulation of OT-I T cells

CD8<sup>+</sup> T cells were purified from the lymph nodes of OT-I and OT-I Drak2<sup>−/−</sup> mice and stimulated in vitro for 2 days with OVA peptide (SIINFEKL) presented by TCRα<sup>−/−</sup> spleenocytes that were first depleted of CD11b<sup>+</sup> cells and mitomycin C-treated. After 2 days, the cells were harvested and stained with annexin V and Abs to CD4 and CD8.

T cell transfer model of colitis

CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>low</sup> (naive) and CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>high</sup> (T regulatory cells) were purified from splenocytes with a FACSaria (BD Biosciences). A total of 3 x 10<sup>6</sup> naive T cells was injected i.v. into RAG1<sup>−/−</sup> mice with or without 2 x 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>high</sup> T regulatory cells. The mice were weighed weekly to monitor the development of colitis, and any mouse that lost 20% of its starting weight was euthanized.

Collagen-induced arthritis

Chicken type II collagen (2 mg/ml; Chondrex) was emulsified with 5 mg/ml CFA (Chondrex), and 100 μl of this emulsion was injected intra-dermally at the base of the tail. Twenty-one days later, a boost of 100 μl of the emulsion was injected intradermally in the back near the base of the tail. The thickness of each paw was determined with a digital micrometer (Fisher Scientific), and the sum of the thicknesses was plotted. In addition, each paw was given a score based on the following scale: 0, normal; 1, slight swelling and/or erythema; 2 extensive swelling and/or erythema; 3, joint distortion and/or rigidity.

Results

Drak2<sup>−/−</sup> mice are resistant to disease in a mouse model of type 1 diabetes

Given the striking resistance to autoimmunity in the induced model of multiple sclerosis, we tested whether the absence of Drak2 would affect disease in other mouse models of autoimmunity. NOD mice spontaneously develop insulitis and diabetes with several features common to human type 1 diabetes, including the involvement of autoreactive CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and pancreatic-specific autoantibodies. In addition, the genes responsible for disease in NOD mice are syntenic to chromosomal regions known to contribute to the predisposition to disease in humans (23).

To determine whether a deficiency in Drak2 inhibited autoimmune diabetes, we backcrossed the B6.Drak2<sup>−/−</sup> mice to NOD.
mice and screened each generation for the 20 NOD susceptibility loci by PCR analysis of microsatellite markers. NOD.Drak2+/− mice that contained all 20 of the NOD susceptibility loci were intercrossed, and littermates were monitored for diabetes by measuring blood glucose levels. Due to the low incidence of disease in males, our analysis only included female mice. Remarkably, NOD.Drak2+/− mice were completely resistant to autoimmune diabetes, whereas the incidence of diabetes in NOD.Drak2+/− and NOD.Drak2−/− mice that were diabetic (two consecutive readings of 300 mg/dL or greater) was plotted. B, The average blood sugar for 19 NOD and 10 NOD.Drak2−/− mice is shown ± SEM.

Drak2−/− mice are susceptible to autoimmunity in mice lacking regulatory T cells

To test whether the absence of Drak2 imparted a generalized immunosuppression and inhibited all types of autoimmunity, we tested the Drak2−/− mice in several models of autoimmune disease. Because autoimmunity can readily occur in the absence of regulatory T cells, we tested two such experimental models: the T cell transfer of colitis (24) and spontaneous encephalitis associated with MBP-specific TCR transgenes (25). To induce colitis, naive (CD45RB+CD25−CD4+) T cells that lack regulatory T cells are transferred into T cell-deficient mice, and disease is indicated by weight loss. If regulatory T cells are transferred along with the naive T cells, the mice do not develop any signs of disease. Naive T cells from both wild-type and Drak2−/− mice induced significant weight loss when transferred into RAG1−/− mice (Fig. 2A). This suggests that the Drak2−/− T cells are able to induce autoimmunity in the absence of regulatory T cells. Moreover, the regulatory T cells from both mice inhibited disease to a similar extent.

The second form of autoimmunity that occurs due to the absence of regulatory T cells involves TCR transgenic mice that express two alleles of a transgenic TCR specific for a peptide from MBP (25). In the absence of regulatory T cells, which was conferred by suppression of endogenous TCR rearrangements, 100% of MBP TCR transgenic mice developed spontaneous EAE (25, 26). Drak2−/− mice were bred to MBP:C57BL/6;1-A+ mice and typed for two copies of the TCR transgene. For comparison, we also tested the effect of the Cbl-b deficiency on this model of autoimmunity. Cbl-b is a negative regulator of T cell activation, and in its absence, T cells are hypersensitive to suboptimal stimulation. However, contrary to Drak2−/− mice, Cbl-b+/− mice are more susceptible to autoimmune disease (8, 9). In this model of EAE, Drak2−/−:MBP+/+ mice developed severe paralysis similar to that in wild-type and Cbl-b−/−:MBP+/+ mice (Fig. 2B). Thus, in the absence of regulatory T cells, Drak2−/− mice are susceptible to autoimmune disease, suggesting that the T cells maintain the potential to become pathogenic. Furthermore, a deficiency in Drak2 does not impact models in which most every T cell bears an autoreactive TCR. In these models in which we saw identical symptoms of autoimmune disease, we did not perform histological analysis.

Drak2−/− mice are susceptible to disease mediated by autoantibodies

We next tested whether Drak2 contributed to forms of autoimmune disease mediated by autoantibodies or cells of the innate immune system. Examples of this are the collagen-induced model of arthritis (27) and a mouse model of systemic lupus erythematosus (SLE) (28, 29). Although T cells are known to play an essential role in the induction of collagen-induced arthritis, their role in the destruction of the joints remains controversial (30). Wild-type and Drak2−/− mice were immunized with type II collagen emulsified in CFA, and monitored for disease by measuring the amount of swelling in the paws. Both wild-type and Drak2−/− mice exhibited similar amounts of joint swelling, as evidenced by the increase in paw thickness (Fig. 2C), and in both groups, all mice immunized experienced joint swelling of at least 0.2 mm, indicating that Drak2−/− mice remain susceptible to disease in this model.

Another autoimmune disease in which the destruction of the tissue is not dependent on T cells is SLE. The B6.NZM.C7 strain of mice is derived from the NZB2410 strain, which spontaneously develops symptoms similar to human SLE, including an increase in activated CD4+ T cells, increased serum levels of IgM and IgG, an increase in the incidence in immune complex glomerulonephritis, and an increase in autoantibodies (29). Drak2−/− mice were crossed with B6.NZM.C7 mice to produce Drak2−/−:B6.NZM.C7 mice. At 18 mo of age, the levels of autoantibodies in the blood and the numbers of activated CD4+ cells in the spleens of littermates were determined. Both Drak2−/−:B6.NZM.C7 and Drak2−/−:B6.NZM.C7 mice had increases in the number of activated CD4+ T cells compared with mice of the same age on a C57BL/6 background (Fig. 2D). In addition, there was an increase in the levels of anti-dsDNA (Fig. 2E) and anti-nuclear Ab (data not shown) in the serum of the B6.NZM.C7 mice compared with the C57BL/6 mice. Thus, the absence of Drak2 did not affect autoimmunity in these models in which the pathogenesis is not dependent on T cells.
Drak2-deficient mice are susceptible to disease dependent on mast cells and neutrophils

The cells of the innate immune system, in particular mast cells (31, 32), can play an important role in inducing inflammation and autoimmune disease. The fact that Drak2−/− mice remained susceptible to colitis, SLE, and collagen-induced arthritis suggests that the inflammatory cells of the innate immune system were functioning; however, we wanted to directly test the function of Drak2−/− mast cells and neutrophils in models that did not involve T and B cells. Thus, we analyzed the function of mast cells in an assay of passive cutaneous anaphylaxis (33), and the function of neutrophils in the KRN -C57BL/6xNOD serum transfer model of arthritis (34). In both assays, Drak2−/− mice exhibited inflammation comparable to wild-type mice (Fig. 2, F and G). Together, these data indicate that Drak2−/− mice are not resistant to all autoimmune diseases. In particular, Drak2−/− mice remain susceptible to diseases caused by the absence of regulatory T cells and diseases mediated by autoantibodies or cells of the innate immune system. We conclude that the absence of Drak2 does not result in a

**FIGURE 2.** Drak2−/− mice remain susceptible to certain models of autoimmune disease. A, CD4+CD25+CD45RBhigh cells were purified from wild-type and Drak2−/− mice and injected into RAG1−/− mice with or without CD4+CD25+CD45RBhigh cells (T regulatory cells (Treg)). The mice were weighed to monitor the development of colitis. The percentage of the starting weight for five mice is plotted ± SEM. These data are representative of two separate experiments. B, MBP−/−;H-2b−, Drak2−/−;MBP−/−;H-2b−, or Cbl-b−/−;MBP−/−;H-2b−/− mice were monitored daily for the following signs of disease and given the indicated scores: 0, no signs of disease; 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, complete hind limb paralysis and partial forelimb paralysis. The average disease score is plotted for 10 MBP−/−;H-2b−/−, 16 Drak2−/−;MBP−/−;H-2b−/−, and 8 Cbl-b−/−;MBP−/−;H-2b−/− mice. C, Six wild-type and Drak2−/− mice on the C57BL/6 background were immunized with chicken type II emulsified in CFA. The average paw thickness ± SEM is depicted for each group, and these data are representative of two separate immunizations with at least five mice per group. All mice exhibited at least a 0.2-mm increase in paw thickness. D, Splenocytes from B6.NZMc7 and B6.NZMc7.Drak2−/− mice at 18 mo of age were stained with Abs to CD4 and CD44. The number of CD4+CD44+ T cells in each mouse is plotted. E, Plasma was isolated from B6.NZMc7 and B6.NZMc7.Drak2−/− mice at 18 mo of age and tested in an ELISA for the presence of anti-dsDNA Abs. F, Wild-type and Drak2−/− mice were injected i.p. with 250 μl of KRN -C57BL/6xNOD serum. The thickness of each paw was measured with a micrometer and averaged together. Each point represents the average of five mice ± SEM. These data are representative of two separate experiments. G, Mice were injected with anti-DNP-IgE intradermally in the right ear and PBS in the left ear. The following day, mice were injected i.v. with DNP-human serum albumin in Evan’s Blue dye. The mice were euthanized 30 min later, and a 6-mm punch was taken from each ear. The punch was incubated overnight in formamide to extract the dye from the ear, and the absorbance of the supernatant was measured. The average absorbance for four to five mice is plotted ± SEM. These data are representative of two separate experiments.
Interestingly, MOG peptide emulsified in CFA, as described previously (2). In-group.

representative of three separate immunizations with at least three mice per disease. We also tested whether T cells were responsible for the resistance to EAE in Drak2 diabetes, we investigated the basis for the resistance to EAE in H11002 mice. We previously demonstrated that positive selection was not entirely T cell specific. Although many current therapies used to treat autoimmune disease. For the most part, Drak2 expression is limited to cells of the lymphoid system, and in particular T and B cells (2); however, the low-level expression in other tissues, including the brain (3, 7), suggested the possibility that the phenotypic effects of deletion were not entirely T cell specific. Although many cells contribute to the pathogenesis of EAE, CD4+ T cells are the major mediators of disease (35). We therefore used an adoptive transfer protocol to determine whether a Drak2 deficiency intrinsic to T cells was sufficient to render mice resistant to disease. We also tested whether T cells were responsible for the increased susceptibility to disease in Cbl-b−/− mice. T cells were purified from the lymph nodes of wild-type, Drak2−/−, or Cbl-b−/− mice and transferred i.v. into TCRα−/− mice that lack endogenous T cells. EAE was then induced by immunization with MOG peptide emulsified in CFA, as described previously (2). Interestingly, TCRα−/− mice that received Drak2−/− T cells did not develop any signs of disease, whereas mice that received wild-type and Cbl-b−/− T cells developed severe disease similar to that observed in intact wild-type or Cbl-b−/− mice (Fig. 3). This suggests that the resistance to EAE in Drak2−/− mice is intrinsic to the T cells.

Responsive MOG-specific T cells are present in Drak2−/− mice

One possibility for the inhibition of EAE in Drak2−/− mice is that these mice lack MOG-specific T cells due to alterations in T cell development. We previously demonstrated that positive selection was enhanced in Drak2−/− mice, and thus, it was feasible that negative selection was also enhanced, effectively deleting the MOG-specific T cells in the thymus. A second alternative was that MOG-specific T cells were present in Drak2−/− mice, but that they did not expand in response to immunization. To address these questions, we tested whether Drak2−/− mice, immunized with MOG and CFA, had MOG-specific T cells in the spleen. Fifteen days following immunization, the cells were harvested and tested for proliferation in vitro in response to varying amounts of MOG peptide. Wild-type and Drak2−/− T cells responded similarly to Ag such that the dose-response curves were virtually identical (Fig. 4). These results establish the presence of MOG-specific T cells in Drak2−/− mice, and demonstrate the ability of these T cells to expand in response to MOG peptide. Furthermore, the fact that proliferation to suboptimal amounts of MOG peptide was similar between wild-type and Drak2−/− mice suggests that all of the high-affinity T cells were not deleted in the Drak2−/− mice.

There are fewer IFN-γ+ and TNF-α+ MOG-specific T cells in Drak2−/− mice

Although MOG-specific T cells were present in Drak2−/− mice, altered cytokine production by these T cells could contribute to the resistance to EAE. A distinct subset of CD4+ T cells was recently identified based upon their production of IL-17, and this subset is responsible for the disease manifestations of EAE (36–39). Therefore, T cells were harvested from the draining lymph nodes 15 days after immunization with MOG and CFA, and stimulated in vitro with MOG peptide for 24 h. The supernatant was removed and analyzed for the production of nine different cytokines, including IL-17. We found that there were no significant differences in the levels of cytokines made by wild-type and Drak2−/− T cells after immunization with MOG and CFA, and stimulated in vitro with MOG peptide for 24 h. The supernatant was removed and analyzed for the production of nine different cytokines, including IL-17. We found that there were no significant differences in the levels of cytokines made by wild-type and Drak2−/− T cells after immunization with MOG and CFA, and stimulated in vitro with MOG peptide for 24 h.
of monensin (to block cytokine secretion), and analyzed intracellular IL-17, IFN-γ, and TNF-α production. Only effector or memory T cells that are specific for MOG will be able to produce cytokines in this short time frame; consequently, there is a high correlation between cytokine-secreting cells and Ag-specific cells as determined by tetramer staining (40). Surprisingly, Drak2−/− mice had a similar number of CD4 T cells that produced IL-17 in response to MOG as wild-type mice; however, there were significantly fewer cells that produced IFN-γ or TNF-α (Fig. 5B). This same pattern was also seen in T cells isolated from the draining lymph nodes (data not shown). These data are surprising because it has been demonstrated that IL-17-producing CD4 T cells are necessary and sufficient for the induction of EAE (36–39), yet Drak2−/− mice, which are resistant to EAE, have IL-17-producing CD4 T cells, but fewer IFN-γ+ and TNF-α+ T cells than wild-type mice. Intracellular cytokine analysis revealed that there was a significant population of CD4+ cells that produced IFN-γ, IL-17, and TNF-α (data not shown), which could account for the fact that there was not a difference in these cytokines in the supernatant after 24 h of culture (Fig. 5A). However, it remains possible that although there are fewer cytokine-producing cells in Drak2−/− mice, these cells secrete more cytokines than wild-type T cells.

The resistance to EAE is not due to a difference in the function or number of regulatory T cells in the absence of Drak2

Although the resistance to EAE in Drak2−/− mice transferred with T cells, it was possible that a higher proportion of Drak2−/− T cells are regulatory T cells compared with wild-type T cells. Alternatively, due to the hypersensitivity of T cells in the absence of Drak2, the regulatory T cells from Drak2−/− mice may suppress effector T cells more efficiently than wild-type regulatory T cells. To determine whether there was enhanced regulatory T cell activity in Drak2−/− mice, we first tested Drak2−/− regulatory T cells in an in vitro suppression assay, and found that the regulatory T cells from Drak2−/− and wild-type mice suppressed the proliferation of wild-type effector T cells with equal effectiveness (Fig. 6A). In addition, there was not a sustained increase in the number or proportion of Foxp3+ T cells in the draining lymph nodes or the spleen of Drak2−/− mice during the course of EAE (Fig. 6B).

Although there was no increase in the number of Foxp3+ T cells in Drak2−/− mice, it was possible that Drak2−/− T cells suppressed effector T cells independent of Foxp3. Thus, we tested whether Drak2−/− T cells could inhibit autoreactive wild-type T cells during EAE. Wild-type and Drak2−/− T cells were injected together into TCRα−/− mice, which were then immunized with MOG and CFA. Mice that received both T cells developed severe disease similar to mice that only received wild-type T cells (Fig. 6C), illustrating that Drak2−/− T cells do not inhibit wild-type T cells and the lack of disease is not dominant. Together, these data suggest that the resistance to EAE in the absence of Drak2 is not due to an increase in the number or function of regulatory T cells.

**T cells do not accumulate in the CNS of Drak2−/− mice after immunization with MOG and CFA**

MOG-specific, CD4+ IL-17+ T cells were present in the periphery of Drak2−/− mice, yet the mice did not develop EAE. However, in order for disease to occur in the MOG-induced model of EAE, T cells must become activated and expand in the lymphoid tissue, and then migrate to the CNS and recruit other inflammatory cells. Previously, we reported that there were no infiltrating cells in sections of spinal cords from Drak2−/− mice 21 days after immunization with MOG and CFA. To obtain a more accurate count of the number of T cells in the CNS during the course of EAE, we harvested the brains from mice at various times during EAE and counted the number of Thy1+ CD4+ T cells that had infiltrated the brain. There was a very small increase in the number of T cells in the brain of Drak2−/− mice early after immunization; however, the T cells did not accumulate as they did in wild-type mice (Fig. 6D).
Thus, Drak2+/− mice have MOG-specific CD4+ T cells that proliferate in the lymphoid tissues after immunization and produce IL-17, but these cells do not accumulate in the CNS.

There is not a general defect in migration of T cells in the absence of Drak2.

There are two likely explanations for the lack of T cells in the CNS of Drak2+/− mice after immunization with MOG and CFA. One possibility is that there is a defect in the migration of T cells to nonlymphoid tissue in the absence of Drak2. The other is that Drak2+/− T cells have the ability to enter the CNS, but these cells are more susceptible to death, and therefore, do not accumulate like wild-type T cells. We first examined the migration of Drak2+/− T cells into nonlymphoid tissue by monitoring the appearance of T cells in the skin as a consequence of a delayed-type hypersensitivity reaction and into the lung following LCMV infection. We found no defect in the ability of Drak2+/− T cells to migrate to the skin or the lung during these immune responses (data not shown).

Because entry into the CNS is a highly specialized process involving a specific set of adhesion molecules and chemokine receptors, including α4β1 integrin, PECAM, LFA-1, and CCR5 (41), we analyzed the level of these molecules on Ag-specific T cells during the course of EAE. Again, we did not detect any significant differences in expression of these molecules between wild-type and Drak2+/− T cells (Fig. 7B).

FIGURE 6. There is not a difference in the function or number of regulatory T cells in the absence of Drak2. A, Naive (CD4+CD25−) T cells were purified from the lymph nodes of wild-type mice, and stimulated with soluble anti-CD3 and mitomycin-treated APC in the presence of increasing numbers of Tregs (CD4+CD25+) that were isolated from either wild-type or Drak2+/− mice. The cells were stimulated in triplicate for 3 days and pulsed with [3H]thymidine for the last 12 h. The average of the three wells is depicted ± SD. This experiment is representative of three separate experiments. B, Mice were immunized with MOG and CFA, and cells were harvested from the spleen and draining lymph nodes of three wild-type and three Drak2−/− mice, and stained for intracellular Foxp3. A representative FACS plot of cells from the draining lymph nodes 15 days after immunization with MOG and CFA is shown. The number in the plots represents the percentage of lymphocyte-gated cells that are CD4+Foxp3+. The graphs depict the percentage of CD4+ T cells that are Foxp3+ in each organ ± SEM. These data are representative of two separate experiments with at least three mice per group. C, TCRα−/− mice were injected with 8 × 106 purified wild-type, Drak2+/−, or a mix of both T cells. After 10 days, the mice were immunized s.c. with MOG and CFA, monitored daily for signs of disease, and given a score based on the scale described previously. The average score for three to four mice is shown, and is representative of two separate experiments.

FIGURE 7. T cells do not accumulate in the brain of Drak2−/− mice during EAE. A, Mice were immunized with MOG in CFA, and the cells from the brain were isolated via a Percoll gradient, counted, and stained for CD4 and Thy1. The graph represents the number of CD4+Thy1+ cells in the brain. These data are representative of three separate experiments with at least two mice per group. B, T cells were harvested from the draining lymph nodes 15 days after immunization with MOG and CFA, and restimulated in vitro in the presence of monensin for 4.5 h. The cells were stained with Abs to the molecules indicated above and intracellular IFN-γ. The mean fluorescence intensity (MFI) of each molecule after electronic gating on CD4+ IFN-γ+ cells is graphed. This is the average MFI for three mice, and is representative of three separate experiments.
Drak2<sup>−/−</sup> T cells enter the CNS in response to viral infection, but are more susceptible to apoptosis

Although Drak2<sup>−/−</sup> T cells express similar levels of adhesion molecules on the cell surface, it was possible that signaling downstream of these receptors was defective in the absence of Drak2. Therefore, we tested whether Drak2<sup>−/−</sup> T cells were capable of entering the CNS in response to viral infection with LCMV. Previously, it was demonstrated that Drak2<sup>−/−</sup> T cells entered the brain after intracranial infection with MHV (22); however, the migration of T cells into the CNS may be mediated by different mechanisms when physical trauma to the CNS is involved. Therefore, we infected mice i.p. with LCMV and counted the number of T cells that migrated into the brain after infection. Interestingly, Drak2<sup>−/−</sup> T cells were capable of entering the brain in response to LCMV infection (Fig. 8A). In fact, the number of Drak2<sup>−/−</sup> T cells on day 9 after LCMV infection was slightly increased compared with wild-type T cells. However, later during LCMV infection, there were fewer T cells in the brain of Drak2<sup>−/−</sup> mice compared with wild-type mice. Although this difference was not statistically significant, the Drak2<sup>−/−</sup> T cells that were present displayed an increased proportion of annexin V binding, suggesting that the T cells were in the process of dying (Fig. 8B). Due to the fact that in vivo, apoptotic cells are engulfed soon after they acquire the ability to bind annexin V, this is likely to be a substantial underestimate of the actual number of apoptotic cells present. It is possible that despite the increased rate of death, we did not observe a significant decrease in cell number due to the fact that there is also increased proliferation in the absence of Drak2. These data clearly indicate that Drak2 is not required for T cells to migrate into the CNS, and suggest that Drak2<sup>−/−</sup> T cells may not accumulate in the brain due to an impairment in survival.

Drak2<sup>−/−</sup> T cells in the CNS during EAE are also more susceptible to apoptosis

Although Drak2<sup>−/−</sup> T cells can enter the CNS during viral infection, the migration and survival of chronically stimulated T cells in the CNS during EAE may be mediated by different mechanisms. Therefore, we analyzed the survival of cotransferred wild-type and Drak2<sup>−/−</sup> T cells within the same brain during EAE. T cells were purified from wild-type (Thy 1.1/1.2) and Drak2<sup>−/−</sup> (Thy 1.1/1.2) mice and injected into TCR<sup>α<sup>−/−</sup></sup> (Thy 1.2/1.2) mice. Two weeks after the transfer of T cells, the mice were bled to confirm equal reconstitution. On average, 1.73% of the cells in the blood were Drak2<sup>−/−</sup> Therefore, we tested whether the stream of these receptors was defective in the absence of Drak2. By the fact that there is also increased proliferation in the absence of Drak2, these data clearly indicate that Drak2 is not required for T cell migration into the CNS, and suggest that Drak2<sup>−/−</sup> T cells may not accumulate in the brain due to an impairment in survival.
from the brains early after immunization because, as demonstrated above, we found that there were similar numbers of wild-type and Drak2/−/− T cells in the brain 5 days following immunization (Fig. 7A). We note that in this model, in which T cells are transferred into TCRα−/− mice, the wild-type T cells do not accumulate to the same levels as in intact wild-type mice, despite the fact that these mice develop EAE. Thus, at the time points analyzed, there were similar numbers of wild-type and Drak2/−/− T cells isolated from the brain. Early in the course of EAE, there was no difference in the proportion of apoptotic wild-type and Drak2/−/− T cells isolated from the same brain (Fig. 8C). Beginning at 9 days following immunization, there were significantly more apoptotic Drak2/−/− T cells compared with wild-type T cells. These data indicate that Drak2/−/− T cells enter the CNS after immunization with MOG and CFA, but are more sensitive to death and therefore do not accumulate and induce disease in a manner comparable to wild-type T cells.

It is possible that Drak2−/− T cells are more susceptible to death after migration to the CNS due to Ag encounter by nonprofessional APC. Alternatively, Drak2−/− T cells that have encountered Ag may be more susceptible to apoptosis, regardless of the location of stimulation. Thus, we wished to determine whether the MOG-specific T cells in the draining lymph nodes during EAE were also more susceptible to cell death. However, identification of the MOG-specific T cells by cytokine expression after restimulation in vitro did not allow us to measure apoptosis of the MOG-specific T cells because cells undergoing apoptosis will not survive restimulation and produce cytokines. Therefore, we analyzed the level of annexin V on activated (CD4+CD69+) T cells in the draining lymph nodes as a surrogate measure for Ag-specific activated T cells. As depicted in Fig. 8D, there was a significant increase in the proportion of apoptotic CD4+CD69+ cells in the Drak2−/− mice compared with wild-type mice. This enhanced sensitivity of activated, Drak2−/− T cells to apoptosis most likely accounts for the decreased number of MOG-specific IFN-γ+ and TNF-α+ T cells in the spleen and draining lymph nodes, and the lack of accumulation of T cells in the brain of Drak2−/− mice during EAE.

The number of cells infiltrating the pancreas is decreased and delayed in NOD.Drak2−/− mice

The data presented to date indicate that Drak2−/− mice are resistant to EAE due to a decreased survival of autoreactive T cells. We thus investigated whether a deficiency in Drak2 inhibited type 1 diabetes in NOD mice in a similar way by preventing the accumulation of autoreactive T cells in the pancreas. The pancreas from prediabetic NOD and NOD.Drak2−/− mice was removed at 9, 12, and 20–22 wk of age, and analyzed for infiltrating cells. The islets from two sections of each pancreas were counted, and the number of islets that displayed no insulitis, peri-insulitis (infiltrate surrounding the islet), or insulitis was recorded. In NOD mice, an average of 30% of islets had perisinusitis or insulitis by 9 wk of age, and this increased to 50% by 12 wk of age, and approached 100% by 22 wk (Fig. 9A). In contrast, only 6% of the islets in the NOD.Drak2−/− mice had perisinusitis or insulitis at 9 and 12 wk of age. Surprisingly, at 22 wk of age, ∼70% of the NOD.Drak2−/− mice displayed peri-insulitis or insulitis, and a significant proportion of the infiltrate consisted of T cells (Fig. 9B). However, the islets that were infiltrated in NOD.Drak2−/− mice had fewer infiltrating cells than the islets of NOD mice (Fig. 9B). It is interesting that such a high proportion of the islets had infiltrate, yet not a single NOD.Drak2−/− mouse became diabetic. These data suggest that similar to the mechanism of resistance in EAE, the absence of Drak2 impacts diabetes by preventing the T cells from accumulating in the pancreas. T cells are able to enter the pancreas in the absence of Drak2, but this number is considerably smaller compared with NOD mice, and does not result in diabetes.

Although there was no difference in the number of regulatory T cells in the absence of Drak2 during EAE, we examined whether there was an increase in regulatory T cells in NOD mice that lacked Drak2. Cells were harvested from the spleen, pancreatic draining lymph nodes, and the pancreas of NOD and NOD.Drak2−/− females between 10 and 12 wk of age. In each organ analyzed, there was no significant increase in the proportion of T...
cells that were Foxp3⁺ in the absence of Drak2 (Fig. 9C). There was a slight increase in the proportion of Foxp3⁺ T cells in the pancreas of NOD.Drak2⁻/⁻ mice compared with NOD mice; however, this was not statistically significant (p = 0.112). It is possible that in older mice this difference would be significant. Although there was an increase in the proportion of Foxp3⁺ T cells in the
pancreas, the total number of regulatory T cells was less due to the fact that there were fewer total T cells in the pancreas of NOD.Drak2−/− mice.

NOD.Drak2−/− T cells display an increased sensitivity to death in the pancreatic draining lymph nodes

Finally, we examined whether the reduction of T cells in the pancreas of NOD mice was due to an increased sensitivity to cell death, similar to T cells in the CNS and draining lymph nodes during EAE. We used an experimental model in which equal numbers of Ag-specific NOD or NOD.Drak2−/− autoreactive T cells were transferred into NOD hosts. This allowed us to monitor the survival, proliferation, and death of a set number of cells. NOD. BDC2.5 TCR transgenic mice express a TCR specific for an islet Ag bound to I-A^q. NOD.BDC2.5 T cells transferred into NOD mice divide and accumulate in the pancreatic lymph nodes where Ag is present; however, if these cells are transferred into NOD.Idd9 congenic mice that are not prone to autoimmune disease, the NOD.BDC2.5 T cells divide, but do not accumulate due to peripheral deletion (42). To determine whether the absence of Drak2 also caused peripheral deletion, CFSE-labeled NOD.BDC2.5 or NOD.BDC2.5.Drak2−/− T cells were transferred into NOD mice, and 4 days later, the proliferation, accumulation, and death of these cells were assessed in the pancreatic draining lymph nodes and the nondraining peripheral lymph nodes. An equal number of NOD.BDC2.5 and NOD.BDC2.5.Drak2−/− T cells was found in the nondraining, peripheral lymph nodes (Fig. 10, A and B), and these showed no evidence of cell division, most likely due to the absence of Ag. In contrast, in the pancreatic lymph nodes where the Ag is presented, both the NOD.BDC2.5 and NOD.BDC2.5.Drak2−/− T cells divided. Strikingly, the NOD.BDC2.5.Drak2−/− T cells did not accumulate in the presence of Ag to the same extent as the NOD.BDC2.5 T cells (Fig. 10, A and B). Furthermore, an increased proportion of NOD.BDC2.5.Drak2−/− T cells in the pancreatic lymph nodes was undergoing apoptosis compared with the NOD.BDC2.5 T cells (Fig. 10, A and B). This suggests that the absence of Drak2 restores peripheral tolerance in NOD mice in part, by causing the deletion of the autoreactive T cells in the draining lymph nodes. It is important to note that in the nondraining, peripheral lymph nodes where Ag is not present, there was not an increase in cell death in the absence of Drak2. This observation indicates that Drak2 is not required for the survival of naive T cells, but rather Drak2 is important for the survival of T cells after Ag encounter.

If Drak2−/− T cells are more susceptible to cell death after antigenic stimulation, then there should be an increase in the proportion of dying Drak2−/− T cells stimulated with Ag in vitro. To test this, wild-type or Drak2−/− OT-I T cells were stimulated in vitro with mitomycin C-treated splenocytes pulsed with OVA peptide. To optimize the recovery of dead cells, the APC were first depleted of CD11b^+ cells. With low amounts of OVA peptide, there was no difference in the amount of apoptosis between wild-type and Drak2−/− T cells (Fig. 10C). However, when the T cells were stimulated with high amounts of OVA peptide, there was a significant increase in the percentage of Drak2−/− T cells that were annexin V^+ compared with wild-type T cells. Together, these data suggest that Drak2−/− T cells are more susceptible to apoptosis after high levels or chronic antigenic stimulation. Presumably, fewer Ag-reactive T cells reach the target organs, and those that do continue to be susceptible to high levels of cell death.

Discussion

Drak2−/− mice exhibit a dramatic resistance to autoimmune diseases, such as EAE and type I diabetes, yet manifest no immuno-

deficiencies. In this study, we found that the absence of Drak2 in T cells confers an enhanced sensitivity to cell death following antigenic stimulation. This increased death of T cells exclusively affects autoimmune models in which T cells are required in the target organ to perpetuate disease (MOG-induced EAE and type I diabetes), and does not alter models in which disease is dependent upon a loss of T regulatory cells (colitis or EAE in MBP TCR transgenic mice) or the production of autoantibodies and inflammatory granulocytes (collagen-induced arthritis or SLE). Importantly, the absence of Drak2 did not affect the survival of naive T cells (Fig. 10) or T cells expanding during an acute infection (2, 22), suggesting that Drak2 is essential for the survival of T cells in situations of chronic autoimmune stimulation.

We propose that there is a tipping point of sensitivity to Ag-driven T cell death. On one side, such as in wild-type mice, strong and chronically stimulated T cells accumulate to eventually produce pathology, whereas, on the other side, as in Drak2−/− mice, the rate of cell death equals or exceeds proliferation such that diseases including EAE and type I diabetes are never evident. Our data further demonstrate that this tipping point is extremely delicate because the differences in T cell survival were subtle at any one point; nevertheless, the cumulative effect was dramatic, ultimately determining the presence or absence of disease. Moreover, we were able to consistently observe this difference in several situations, including T cells in the brain and draining lymph nodes following immunization with MOG and CFA, activated T cells in the draining lymph nodes in NOD mice, and T cells stimulated in vitro with high levels of Ag. It is remarkable that a 2-fold increase in cell death can give rise to such a significant difference in T cell number in the brain during MOG-induced EAE and the pancreas of NOD mice. However, in a situation of exponential accumulation, a 2-fold increase in cell death at each round of division correlates to an 8-fold difference in cell number after only three rounds of division. Furthermore, the increase in dying cells that we detected in vivo is almost certainly an underestimate of the actuarial rate of death that occurs due to the fact that dying cells are rapidly engulfed by macrophages. The concept that enhanced T cell apoptosis results in less autoimmune disease is appreciated in a number of models of EAE (43–52) and in the NOD model of type I diabetes (53–56). Conversely, a decrease in T cell apoptosis can exacerbate EAE (57–62) or diabetes (63–65). Of note, the transgenic expression of Bcl-2 did not affect the acute phase of EAE, but rather caused a more severe chronic phase of the disease (57).

The notion is that the survival rate of T cells is especially important under conditions of chronic antigenic stimulation. Thus, it is clear that modulating apoptosis of activated T cells has a significant impact on these diseases, and Drak2 is yet another factor that regulates T cell survival and subsequently affects autoimmune disease.

The fact that activated T cells are more prone to death in the absence of Drak2 raises the question of why Drak2−/− mice remain susceptible to disease in some models of autoimmune disease. Both the collagen-induced model of arthritis and the mouse model of SLE require T cells in the initial priming of the autoimmune response; however, autoantibodies and other inflammatory cells may play a more critical role in the pathogenesis of disease. Thus, although the activated T cells may be more susceptible to death in this situation, it does not impact disease because the T cells are dispensable at this point. In contrast, T cells are required for the pathogenesis of both colitis and the spontaneous model of EAE, yet Drak2−/− mice are susceptible to autoimmunity in these models. In both of these models, disease is induced only in the absence of regulatory T cells. Perhaps, under these conditions, fewer autoreactive T cells are required to induce disease, or the
time to disease manifestation is decreased. Although the autoreactive Drak2−/− T cells maintain an increased sensitivity to death, these cells are not completely eliminated, and in the absence of regulatory T cells, the remaining T cells are sufficient to induce disease. Furthermore, in the spontaneous model of EAE that involves MBP TCR transgenic mice, every single T cell possesses an autoreactive TCR, and to prevent disease nearly every T cell would have to be eliminated.

The fact that the absence of Drak2 does not affect autoimmunity in which T cells are required in the initiation of disease suggests that Drak2 does not affect the survival of T cells early in the progression of disease, but rather during a phase of chronic antigenic stimulation. In fact, the expansion and survival of Drak2−/− T cells were completely normal in several situations of acute stimulation, including immunization with OVA and LPS (data not shown), infection with LCMV (2), infection with L. monocytogenes (data not shown), and MHV infection (22).

The primary phenotype exhibited by Drak2−/− T cells is enhanced sensitivity to TCR-mediated stimulation, yet the cells die at an increased rate under conditions of chronic autoimmune stimulation. We propose that Drak2 affects the survival of T cells in situations where there is persistent TCR-mediated stimulation, but little inflammation, such as during an autoimmune response or a chronic infection. In the presence of acute infection resulting in overt inflammation, the level of costimulatory molecules and cytokines is high and provides survival signals that can balance the high levels of TCR stimulation. Alternatively, under conditions in which costimulation is minimal, the highly stimulated Drak2−/− T cells die for lack of survival signals conveyed by stimulation or cytokines. This could explain why intracranial infection with MHV in Drak2−/− mice induced the migration of a similar number of T cells into the CNS as in wild-type mice, and there was no effect of a Drak2 deletion on either the survival of T cells or subsequent demyelination (22). It is likely that intracranial infection with MHV induces significantly more inflammation directly in the CNS compared with immunization in the hind flank with MOG and CFA, and this increased inflammation produces costimulatory molecules or cytokines that preclude the requirement for Drak2. In support of this, after i.p. infection with West Nile virus, there were fewer T cells and a lower viral load in the brain of Drak2−/− mice compared with wild-type mice. However, intracranial infection resulted in a similar viral load in the brain in both strains of mice (66). Thus, we propose that long-term T cell survival depends on a balance of TCR-mediated signaling and survival signals. Strong TCR signaling, such as occurs with therapeutic anti-CD3 treatment (67) without balancing costimulation, causes T cell death. This makes Drak2 an attractive target to treat autoimmunity because inhibition should not affect the response to acute infections.

From a broader viewpoint, it would seem that there is no apparent selective value to maintaining chronically stimulated T cells, because this is typically restricted to two situations, as follows: infection by agents that are rarely transmitted and thus maintained at a state of chronic contagion (examples include many venereal diseases or vertically transmitted diseases), and autoimmune-hypersensitivity diseases, in which Ag is persistent because it is self encoded or pervasive in the environment. In either case, it is difficult to discern the selective value in maintaining activated T cells, and thus the present selective value of Drak2. As a guess, perhaps there is a disease or a class of diseases in which long-term enhanced survival of Ag-specific T cells is advantageous. A characterization of the signaling mediated by Drak2 will aid in our understanding the balance of signals needed for T cell survival and autoimmune disease development.

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