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Enhanced T cell apoptosis within *Drak2*-deficient mice promotes resistance to autoimmunity[1]

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Abstract

Clonal expansion of T cells is vital to adaptive immunity, yet this process must be tightly controlled to prevent autoimmune disease. The serine/threonine kinase DRAK2 is a negative regulator of T cell receptor (TCR) signaling and sets the threshold for the activation of naïve and memory T cells, and selected thymocytes. Despite enhanced T cell activation, *Drak2*−/− mice are resistant to experimental autoimmune encephalomyelitis (EAE), an autoimmune demyelinating disease that resembles multiple sclerosis. However, the basis for this autoimmune resistance is currently unknown. Here we show that, in the absence of DRAK2 signaling, T cells require greater tonic signaling for maintenance during clonal expansion. Following stimulation, *Drak2*−/− T cells were more sensitive to an intrinsic form of apoptosis that was prevented by CD28 ligation, homeostatic cytokines, or enforced Bcl-xL expression. T cell-specific Bcl-xL expression also restored the susceptibility of *Drak2*−/− mice to EAE and enhanced thymic positive selection. These findings demonstrate that DRAK2 is selectively important for T cell survival and highlight the potential that DRAK2 blockade may lead to permanent autoimmune T cell destruction via intrinsic apoptosis pathways.

Keywords

Apoptosis; signal transduction; autoimmunity; experimental autoimmune encephalomyelitis; superantigen

Introduction

Several parameters regulate a T cell's decision to proliferate, including amount and avidity of TCR stimulation, presence of costimulation, and duration of T cell:APC interactions (1–4). Peripheral T cell tolerance to self is, in part, mediated through low avidity interactions between the T cell receptor (TCR) and self-antigen presented by non-activated APCs which lack sufficient expression of costimulatory molecules, resulting in T cell anergy, clonal deletion, or T cell suppression (4–7). When these signals are disrupted, the threshold for T cell activation is altered, often allowing inappropriate initiation of T cell responses to self-antigen and

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ultimately resulting in autoimmune disease. Consistent with this, exacerbated autoimmune susceptibility is a common phenotype observed in mice lacking negative regulatory molecules such as Cbl, GRAIL, MGAT5, and PTEN (8–12).

After an infection is cleared, most T cells generated during the clonal expansion phase are removed through either of two apoptotic pathways termed activation-induced cell death (AICD) and activated cell autonomous death (ACAD) (13). AICD is thought to depend on an extrinsic form of apoptosis induced by the ligation of death receptors such as Fas on the surface of the T cell (14). Patients with mutations in Fas display increased survival of lymphocytes and develop a disease termed Autoimmune Lymphoproliferative Syndrome (ALPS) (15). Also, mice with mutations in Fas (*lpr*) or FasL (*gld*) develop lymphadenopathies and an accumulation of autoreactive T cells in the periphery, indicating the importance of Fas-dependent AICD in lymphocyte homeostasis as well as peripheral tolerance (16,17).

While many of the molecular mechanisms behind AICD have been elucidated, much less is known about ACAD. ACAD is thought to depend on an intrinsic form of cell death similar to that induced by cytokine withdrawal. This form of "death by neglect" is regulated by the balance of pro- (Bim, Bad, Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL, IAPs) factors, which themselves are subject to control by tonic signaling (18). Cytokine withdrawal results in reduced transcription of Bcl-2, Bcl-xL, and the cIAP family of caspase inhibitors (14,15,19). Reduced levels of these proteins relieve inhibition of Bim and Bad, allowing activation of Bax and Bak which can go on to initiate a mitochondrial form of apoptosis. T cells overexpressing Bcl-2 or Bcl-xL are resistant to this form of death, whereas Bim sensitizes activated T cells to apoptosis following viral clearance (20–22).

Factors that regulate T cell proliferation have also been shown to influence subsequent AICD and ACAD. Strong TCR stimulation induces genes not only involved in proliferation, but also in cell death such as Fas, FasL, and Bim (23,24). Prolonged culture in high IL-2 concentrations leads to decreased expression of the caspase inhibitor c-FLIP, thus sensitizing these proliferating T cells to apoptosis (23). Recent evidence suggests that strong TCR stimulation not only initiates proliferation, but also encourages T cells to undergo AICD. For example, T cells from mice lacking an inhibitor of calcineurin, calcipressin (Csp1), succumb to enhanced induction FasL surface expression in response to TCR ligation, resulting in a reduced proliferative capacity due to exacerbated AICD (25). While costimulatory signals clearly reduce the activation necessary for the commitment of naïve T cells to clonal expansion, signals derived via costimulatory receptors are also crucial for activated T cell survival. CD28, 4-1BB and OX40 promote activated T cell survival through sustained activation of Akt which induces Bcl-xL and Bcl-2 expression (26–33). Additionally, active Akt may also serve to limit apoptotic signaling by directly phosphorylating and inactivating Bad, as well as by interfering with the function of FOXO factors (34,35). The latter may be crucial for dictating the apoptotic sensitivity of T cells, since a number of proapoptotic genes, including Bim, are transcriptionally regulated by FOXO factors (35,36). It is clear that the coordination of survival and proliferative signaling, while highly complex, is essential for maintaining immune homeostasis and tolerance (37).

DRAK2 (death-associated protein kinase (DAPK)-related apoptosis-inducing kinase 2) is a serine/threonine kinase distantly related to the DAP-kinase family, a group of serine/threonine kinases thought to potentiate apoptosis (38). However, a direct role for DRAK2 in modulating apoptosis is a matter of controversy; ectopic expression of DRAK2 has been shown to induce apoptosis, but *Drak2*−/− mice display no discernable apoptotic deficits (38–41). Although expressed at low levels in various tissues (42,43), DRAK2 expression is highly enriched in lymphoid organs (40). DRAK2 negatively regulates TCR-mediated calcium mobilization, IL-2 production and restricts clonal expansion following weak TCR stimulation. Consistent with

these findings, *Drak2*−/− T cells initiate a proliferative response following suboptimal costimulation (40). However, unlike mice lacking other negative regulators of T cell activation, *Drak2*−/− mice do not develop overt signs of spontaneous autoimmune disease and display resistance to experimental autoimmune encephalomyelitis (EAE) following myelin oligodendrocyte (MOG) peptide immunization (40,44). Given the paradoxical finding that mice lacking DRAK2 are refractory to autoimmune diseases but possess hyperactive T cells, we hypothesized that DRAK2 may serve to maintain the survival of proliferating T cells. Here, we provide evidence that heightened T cell apoptotic sensitivity leads to autoimmune resistance in *Drak2*−/− mice, suggesting that DRAK2 is an essential mediator of immunological tolerance.

Materials and Methods

Mice, antibodies, and reagents

Drak2−/− mice bred onto the C57BL/6J background were generated as previously described (40). *Bim*−/− mice were obtained from Jackson Labs. Lck^{pr}-Bcl-xL transgenic mice were obtained from the laboratory of Craig Thompson (22). Antibodies against CD4 (APC, biotin), CD8 (biotin), B220, MHCII, CD11b, CD3, CD28, CD25, and IL-2 (clones JES6-1A12 and JES6-5H4), and streptavidin-PerCP were from eBioscience. Annexin-V (PE, APC), and antibodies against CD4 (PE), CD8 (FITC), and CD69 were from Caltag Laboratories. Antibodies against CD8 (PerCP), Vβ7 (biotin), Vβ8 (PE, FITC, biotin), were from Pharmingen. SEB was from Toxin Technologies. The anti-DRAK2 mAb was purchased from Cell Signaling Technologies (Danvers, MA).

Cell purification

Splenic T cells were purified by negative selection using biotinylated antibodies against B220, MHC-II, and CD11b followed by separation using streptavidin-conjugated magnetic beads (Miltenyi Biotech). Typical purity was greater than 95%. Wildtype splenic APCs were purified by depletion of T cells using biotinylated antibodies against CD3, CD4, and CD8 followed by separation using streptavidin-conjugated magnetic beads.

Thymidine incorporation, cell recovery, and IL2 production

Purified T cells were cultured in round-bottom wells with purified, mitomycin C-treated wildtype APCs and various concentrations of SEB. Proliferation was measured in triplicate by ^{3}H-thymidine incorporation during the last 18h of culture. Cell recovery was measured by timed FACS collection of cells stained with biotinylated anti-Vβ7 and anti-Vβ8/streptavidin-PerCP. IL-2 levels of supernatants were measured in triplicate by ELISA.

Intracellular Staining

For Bcl-xL expression, purified T cells activated 6 days with SEB were surface stained with biotinylated anti-Vβ8/streptavidin-PerCP, then permeabilized with a Cytofix/Cytoperm kit according to the manufacturer's directions (BD Biosciences). Cells were then stained with either Bcl-xL (Cell Signaling) or isotype control antibodies then analyzed by FACS. For determining expression of IFNγ and IL-17, T cells were harvested from the spleens of mice 14d post-immunization with $MOG_{35–55}$ emulsified in CFA, and magnetically sorted $CD4^{+}$ T cells were stimulated with PMA plus ionomycin for 6h in the presence of GolgiPlug (BD Biosciences). Cells were stained with anti-CD4-APC, fixed and permeabilized as above, and then stained with anti-IFNγ-PE or anti-IL-17-PE. For intracellular DRAK2 levels, thymocytes from wildtype and Drak2−/− mice were harvested, stained with anti-CD4-APC and anti-CD8-PE, followed by permeabilization as above. Anti-DRAK2 was added for 30 min, followed by washing and staining with FITC-conjugated anti-rabbit Abs.

Quantitative real-time RT-PCR (QPCR)

Purified splenic T cells from either *Drak2*-deficient mice or wildtype littermates at a density of 10^6/ml were activated with plate-bound anti-CD3ε (1 μg/ml for optimal or 0.1 μg/ml for suboptimal stimulation) in the presence or absence of 1 μg/ml soluble anti-CD28 for either 24 or 48 hours and subsequently expanded in the presence of IL-2 (100 U/ml). Following isolation of total RNA with TRIzol solution, cDNA was generated from 1 ug total RNA for each timepoint and condition using the Superscript First-Stand Synthesis System with oligo(dT) primers (Invitrogen). Samples were analyzed in triplicate by QPCR with an iCycler using the iQ™ SYBRGreen Supermix and specific primers for 40 amplification cycles. After normalization of all data to β-Actin, fold changes were calculated by dividing the values for stimulated by unstimulated samples.

Retroviral Infections

Murine stem cell virus IRES GFP (Mig) vectors encoding Bcl-xL were kind gifts from Michael Croft. The Ψ-eco packaging vector was a kind gift of Owen Witte. Retrovirus was collected from the supernatants of calcium phosphate transfected 293T cells and titered by GFP expression of infected 3T3 cells. T cells were infected by centrifugation for 90 minutes at 1800 rpm in the presence of retrovirus and 4 μg/ml polybrene at 48 and 72 hours following activation with SEB. 18 hours later retroviral supernatant was replaced with fresh media and cells were left to culture for a total of 8 days. Cells were then collected and stained with biotinylated anti-Vβ7 & anti-Vβ8/streptavidin-PerCP for FACS analysis.

Experimental Autoimmune Encephalomyelitis and histology

Mice were challenged with $MOG_{35–55}$ as previously described (40). Mice were immunized with 125 μg $MOG_{35–55}$ (prepared in the laboratory of Prof. Charles Glabe, University of California, Irvine) emulsified in complete Freund's adjuvant containing H37Ra mycobacterium tuberculosis (Fisher Scientific, Tustin, CA) in each hind flank on days 0 and 7. Mice also received intraperitoneal injections of 200 ng Bordetella pertussis toxin (List Biologicals, Campbell, CA) in sterile PBS on days 0, 2, and 7. Mice were then monitored for signs of disease on the indicated days. Clinical disease following EAE induction was assessed using a previously described scale (40) as follows: (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 fore limb paralysis, [5] death. Spinal cords and brains were removed at 25d post-immunization. Spinal cord sections were fixed by immersion in 10% normal buffered formalin for 24 hours for paraffin embedding. The severity of demyelination was determined by luxol fast blue staining of spinal cords and analyzed by light microscopy (45). Frozen brain sections were stained with Alexa-488- conjugated anti-CD3 (clone 2C11), followed by light microscopy.

Bim Western Blotting

Wildtype and *DRAK2*$^{-/-}$ T cells were cultured with mitomycin C treated-wiltype APCs pulsed with 2 μg/ml of SEB. On days 0, 3, and 6 T cells were harvested, cleared of non-viable cells using Ficoll, and then magnetically sorted for Vβ7/8 expression using magnetic beads. Vβ7/8 purified T cells were lysed in complete lysis buffer (150 mM NaCl, 50 mM sodium fluoride, 10 mM β–glycerophosphate, 20mM HEPES pH 7.4, 1% Triton X100 with 1mM sodium vanadate, 1 mM phenylmethylsulphonylfluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin), and lysates were resolved on a 12% SDS-PAGE gel. The proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA), pre-washed in Tris buffered saline/tween 20 (TBST), then blocked in TBST supplemented with 5% BSA and incubated with anti-BIM/BOD purified polyclonal antibody (Imgenex, San Diego, CA, USA) at a 1:1000 dilution. Following an overnight incubation with the primary antibody, the blot was washed three times

in TBST, and incubated with peroxidase-labeled anti-rabbit secondary antibody (Vector Labs, Burlingame, CA, USA) for a 1-hour incubation at room temperature. To visualize western, the blot was washed three times in TBST, then incubated in ECL reagent (Amersham Biotech) and exposed to X-ray film.

Results

Enhanced death of Drak2−/− T cells following superantigen stimulation is blocked by tonic signaling through Bcl-xL

To investigate the potential that DRAK2 may contribute to survival signaling, purified *Drak2* −/− or wildtype T cells were stimulated *in vitro* with the superantigen staphyloccal enterotoxin B (SEB) in the presence of wildtype, mitomycin C-treated antigen presenting cells (APCs), then analyzed for proliferation and SEB-reactive (Vβ7/8 TCR^+) T cell recovery (46). *Drak2* −/− T cells had both decreased proliferation and live cell recovery in response to SEB (Fig. 1A, B). This was not due to intrinsic defects of the SEB-reactive pool in *Drak2*$^{-/-}$ mice since the proportion of $V\beta8^+$ T cells, as well as the CD4:CD8 ratio within this pool, was similar to wildtype mice (Supp. Figs. 1A, B). Also, SEB-activated *Drak2*−/− T cells upregulated activation markers and produced IL-2 to a similar extent as wildtype T cells, excluding decreased activation or distinct TCR repertoire pools in *Drak2*−/− mice as a cause of reduced SEB-mediated proliferation (Supp. Figs. 1C, D). To determine whether this decreased proliferation and recovery was due to enhanced apoptosis, purified T cells were CFSE-labeled and subsequently analyzed by FACS after 8d of SEB culture. Recovery of live proliferating *Drak2*−/− T cells was significantly reduced following SEB stimulation (Fig. 1C, left panel). A greater proportion of dividing *Drak2*−/− T cells were annexin-V^{Hi}, demonstrating that the majority of these T cells responded to SEB by proliferating and subsequently undergoing apoptosis (Fig. 1C, right panel). *Drak2*−/− T cell death following superantigen stimulation was Fas-independent, since treatment with Fas:Fc failed to restore clonal expansion (Fig. 1D) or survival of these cells (data not shown).

That FasL blockade failed to rescue abortive proliferation in this context was consistent with previously published data indicating that superantigen-mediated T cell apoptosis occurs via a death receptor-independent pathway. Rather, this death is mediated by control over the relative expression of pro-survival and pro-apoptotic Bcl-2 family proteins (14,18,47,48). This form of death can be prevented by immunological adjuvants and costimulatory survival signals which promote sustained Bcl-2 and Bcl-xL expression (27,31,32,49–53). To determine whether costimulation might restore *Drak2*−/− T cell survival, purified T cells were stimulated with SEB in the presence of agonistic anti-CD28 to mimic enhanced costimulatory conditions. Exogenous CD28 ligation restored *Drak2*−/− T cell proliferation and live cell recovery in response to SEB (Fig. 2A), and this rescue was most profound for high-dose superantigen stimulation (Fig. 2B). CD28 not only promotes survival of activated T cells, but amplifies TCR signaling and subsequent T cell activation (54). Indeed, anti-CD28 enhanced SEB-mediated IL-2 production by both wildtype and *Drak2*−/− T cells (Supp. Fig. 2), although exogenous IL-2 did not promote *Drak2*−/− T cell survival (data not shown). However, anti-CD28 reduced the proportion of dividing *Drak2*−/− T cells that were annexin-V^{Hi} (Fig. 2C and D). Surface expression of CD28 was indistinguishable between wildtype and *Drak2*−/− T cells following SEB activation for 8d (Supp. Fig. 3), demonstrating that the increased costimulatory requirement in the mutant T cells is not due to diminished CD28 expression. Addition of the homeostatic cytokines IL-7 and IL-15 also restored *Drak2*−/− T cell survival (Fig. 2E), suggesting that *Drak2*−/− T cells have a greater dependence on survival factors to prevent their apoptotic demise following SEB activation. Addition of blocking Abs to IL-7 and IL-15 failed to block anti-CD28 mediated rescue of SEB-stimulated *Drak2*−/− T cells (data not shown),

suggesting that such tonic signaling may occur independently via cytokine or costimulatory receptors.

CD28-mediated costimulation and IL-7/IL-15 all promote T cell survival through the upregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (31,37,55). Using quantitative realtime PCR, we observed that anti-CD3 stimulated *Drak2*−/− T cells displayed diminished levels of Bcl-xL following 6d culture in IL-2 when compared to wildtype T cells, although levels of expression of Bcl-xL after 24h were indistinguishable (Fig. 3A). Similarly, we found that *Drak2*−/− T cells stimulated for 6d with SEB-pulsed APCs failed to maintain Bcl-xL expression, and this was restored to wildtype levels by addition of anti-CD28 (Fig. 3B). Consistent with diminished Bcl-xL expression, we observed a greater proportion of $DiOC6^{Lo}$/$hydroethedium^{Hi}$ *Drak2*−/− T cells following mitogenic stimulation (Figs. 3C, D), a probable consequence of mitochondrial disruption known to occur during intrinsic apoptosis (56). To determine whether expression of Bcl-xL was sufficient to promote *Drak2*−/− T cell survival, SEB-activated T cells were infected with a MIG retrovirus encoding the infection marker GFP with or without Bcl-xL, and then collected on day 8 for FACS analysis. Because cell division is required for infection by MSCV-based retroviruses, the relative percent of live-gated GFP^+ cells recovered was used as an indicator of proliferating T cell survival. Retrovirus-enforced expression of Bcl-xL rescued SEB-responsive *Drak2*−/− T cell recovery (Fig. 3E,Supp. Fig. 4), consistent with the hypothesis that *Drak2*−/− T cells are hypersensitive to intrinsic apoptosis following superantigen stimulation. As an alternative means to validate this hypothesis, *Drak2*−/− mice were bred with a transgenic mouse line in which Bcl-xL is constitutively expressed in T cells (Lck^{pr}-Bcl-xL Tg), and also with mice deficient in the pro-apoptotic Bcl-2 family member Bim (22,57). Previous studies using these mouse lines have demonstrated the rescue of T cells from activated cell death (20,31,47). Importantly, Bim has been shown to be essential for SEB-induced T cell death (47). Although transgenic Bcl-xL expression enhanced the recovery of wildtype T cells, there was even greater recovery of *Drak2* −/− x *Bcl-xL* Tg T cells following SEB stimulation (Fig. 3F), supporting our findings with retrovirus enforced Bcl-xL expression. A Bim deficiency improved the recovery of wildtype and *Drak2*−/− T cells to a similar extent, consistent with the role of this BH3-only proapoptotic Bcl-2 homolog in promoting T cell apoptosis (13). However, Bim expression was indistinguishable between wildtype and *Drak2*−/− T cells following SEB stimulation (Fig. 3G). This finding suggests that Bim contributes to the apoptotic hypersensitivity of *Drak2*−/− T cells, but that its expression is not likely a target of DRAK2 activity. Taken together, these results demonstrate that *Drak2*−/− T cells are apoptotically hypersensitive and require heightened tonic signaling to maintain their survival.

Blockade of apoptosis by T cell intrinsic Bcl-xL expression restores EAE sensitivity to *Drak2* −/− mice

Drak2−/− mice are resistant to EAE, and display a profound lack of T cells recruited to the central nervous system (CNS) 21 days following MOG-peptide immunization (40). Our results with SEB suggested that enhanced T cell instrinsic apoptosis might account for the resistance of *Drak2*−/− mice to this autoimmune disease. To test this hypothesis, EAE was induced in wildtype, *Drak2*−/−, *Bcl-xL* Tg, and *Drak2*−/− x *Bcl-xL* Tg mice by immunization with $MOG_{35–55}$ peptides plus complex Freund's adjuvant (40,44). Consistent with previous results, *Drak2*−/− mice had diminished clinical scores following MOG peptide immunization, with two independent experiments shown (Figs. 4A, B). While transgenic Bcl-xL expression did not significantly alter disease severity in an otherwise wildtype background, this transgene completely restored the susceptibility of *Drak2*−/− mice to EAE. Since the Bcl-xL transgene is T cell specific, these results not only implicate apoptotic hypersensitivity as a mechanism of resistance of *Drak2*−/− mice to EAE, but also demonstrate that this resistance is T cell intrinsic. Consistent with this, we observed restoration of $CD3^+$ T cells in the central nervous

systems of $MOG_{35–55}$ immunized *Drak2*−/− mice and enhanced demyelination upon expression of the Bcl-xL transgene (Fig. 4C, D). We also observed a defect in the maintenance of $MOG_{35–55}$ reactive CD4+ T cells as assessed by *ex vivo* culture of splenic T cells with antigen-pulsed APCs following 7d of MOG immunization (Fig. 5A). Immunization of *Drak2* −/− mice failed to give rise to increased ratios of IFNγ– or IL-17 expressing $CD4^+$ T cells (Figs. 5B, C), although *Drak2*−/− mice have unimpaired differentiation of either Th1 (40,44) or Th17 (Fig. 5D) subsets following culture under biasing conditions. These findings suggest that the maintenance of MOG-reactive Th1 and Th17 cells, subsets known to be required for EAE (58), is dependent upon DRAK2. Consistent with this, we observed that the Bcl-xL transgene restored an increased fraction of MOG-reactive IFNγ-expressing Th1 clones following 14d of *in vivo* MOG peptide challenge (Fig. 5E). While we did not observe significant restoration of MOG-reactive Th17 cells by the Bcl-xL transgene in *Drak2*−/− spleens (Fig. 5F), it may be that such cells were instead recruited to the CNS to promote EAE, or that rescued anti-MOG Th1 cells may have supplanted the need for Th17 cells (59). Taken together, our data provide evidence that DRAK2 plays an essential role in maintaining the survival capacity of MOG-reactive T cells *in vivo* during EAE.

Enhanced thymocyte positive selection in *Drak2*−/− mice revealed by a Bcl-xL transgene

Given that DRAK2 serves to control the activation and survival of peripheral T cells, we turned our attention to thymocyte selection to determine if an analogous duality in its function may be present during thymopoiesis. In previous studies, we observed a role for DRAK2 in setting the threshold for TCR-induced Ca^{2+} mobilization in double positive (DP) thymocytes during selection (60). Consistent with this, intracellular staining with monoclonal anti-DRAK2 Abs revealed predominant expression in thymic subsets undergoing, or having completed selection (Fig. 6A and Supp. Fig. 5). Namely, we observed high levels of DRAK2 in single positive and transitional T cells. Elevated DRAK2 expression was also observed in $CD4^{Lo}/CD8^{Lo}$ thymocytes, a population thought to represent thymocytes undergoing selection (61). While *Drak2*−/− mice displayed modestly enhanced thymocyte positive selection when bred with AND and OT-II transgenic mice, no obvious differences in positive selection were observed in non-TCR transgenic backgrounds and negative selection was not apparently impacted by this deficit (40). Since TCR signal strength is a fundamental determinant for the outcome of DP thymocyte selection, we wished to establish whether the absence of an enlarged population of positively selected thymocytes in non-TCR transgenic *Drak2*−/− mice might be due to the apoptotic demise of such cells. To assess this, we evaluated the thymic CD4 vs. CD8 phenotypes of *Drak2*−/− mice with or without the Bcl-xL transgene. While *Bcl-xL* transgenic mice possessed a significant increase in the proportion of CD8 single positive thymocytes, we observed a dramatic increase in the proportion of both CD4 and CD8 single positive thymocytes derived from *Drak2*−/− x *Bcl-xL* mice in five separate experiments, with representative data shown for littermates of the indicated genotypes (Fig. 6B). We failed to observe significant differences in thymus size or cellularity between littermates in these studies (data not shown). While the Bcl-xL transgene led to enhanced proportions of both $CD4^+$ and $CD8^+$ peripheral T cells, the absence of DRAK2 did not appear to affect these peripheral lymphocyte numbers (Fig. 6C). These data suggest that DRAK2 restricts thymocyte selection by negatively regulating TCR signaling, and that in its absence, selected T cells are hypersensitive to apoptosis. Given that no major differences in $CD4^+$ and $CD8^+$ peripheral T cell populations were observed beyond those provided by enforced Bcl-xL expression, our results also reveal that DRAK2 likely does not control the maintenance of peripheral naïve T cells.

Discussion

Our studies indicate that while DRAK2 blocks T cell activation (40,60,62), this lymphoid-enriched serine-threonine kinase also plays an essential role in maintaining the survival of T

cells activated under specific contexts. We have found that *Drak2*−/− T cells are more susceptible to intrinsic apoptosis, suggesting that DRAK2 activity promotes the survival of T cells during clonal expansion following antigenic stimulation. Consistent with these findings, we observed diminished T cell survival following antigenic stimulation with SEB and with MOG peptides. Since SEB- and MOG-reactivity were restored by a Bcl-xL transgene, our results are in accord with the hypothesis that DRAK2 signaling impacts the balance in expression of pro-survival vs. pro-apoptotic Bcl-2 family members during the course of a T cell response to these antigens.

Although a DRAK2 deficiency leads to defective survival of MOG-reactive cells in EAE, such mice have no significant defects in immunity to acute viral infection. *Drak2*−/− mice display overtly normal antiviral T cell responses to lymphocytic choriomeningitis virus (LCMV) and murine hepatitis virus (MHV), and generate antiviral memory T cells (40,44,63). While the bases for the differences in apoptotic hypersensitivity are currently unclear, we hypothesize that necessity for DRAK2 signaling in peripheral T cells may be dependent upon the inflammatory context or nature of the antigen. Unlike an antiviral response in which pathogen associated molecular patterns (PAMPs) elicit an array of costimulatory survival molecules and inflammatory cytokines, EAE results from an autoimmune response against self-antigen induced in the absence of sustained pathogenic “danger signals” (64,65). It is therefore possible that *Drak2*−/− T cells do not receive sufficient costimulatory- or cytokine-mediated survival signals within the CNS during EAE development to maintain their survival. Consistent with this, transgenic Bcl-xL expression restored susceptibility of *Drak2*−/− mice to EAE. Additionally, *Drak2*−/− mice develop neuroinflammatory disease indistinguishable from *Drak2*+/+ mice following neurotropic mouse hepatitis virus (MHV) infection, a model in which chronic viral infection promotes sustained and/or enhanced costimulatory ligand and inflammatory cytokine expression within the CNS (44,66–68). Although there may be a variety of alternative explanations for the differential requirements for DRAK2 signaling in anti-viral vs. autoimmune settings, we have not observed increased apoptotic sensitivity of *Drak2*−/− T cells responding to MHV or LCMV. Thus, it is likely that the apoptotic hypersensitivity of *Drak2*−/− T cells is limited by tonic factors present in an antiviral context.

Previous work has established that DRAK2 restricts TCR-dependent signaling in developing thymocytes, peripheral T cells, and in antiviral memory $CD8^+$ T cells (40,60,63). Here, we have demonstrated that DRAK2 also serves to maintain the survival of activated T cells and selected thymocytes. Expression of a Bcl-xL transgene in *Drak2*−/− mice led to restoration of MOG-induced EAE, and enhanced the recovery of positively selected thymocytes. Given the enhanced recovery of positively selected thymocytes in *Drak2*−/− *x Bcl-xL* mice, it is possible that the restoration of EAE sensitivity observed might be due to enlarged populations of peripheral T cells in these mice. However, this explanation is unlikely since the Bcl-xL transgene only modestly affected the recovery of naïve peripheral $CD4^+$ T cells, the subset that responds to $MOG_{35–55}$. While a DRAK2 deficiency during thymopoiesis may very well alter the repertoire of the peripheral T cell pool, we suggest that DRAK2 also plays an important role in peripheral T cell survival. This hypothesis is strengthened by the finding that retrovirus-enforced expression of Bcl-xL rescued peripheral T cell responses to SEB. Thus, the restoration of EAE sensitivity by the Bcl-xL transgene likely reflects enhanced survival of peripheral effector T cell populations. Recently, it has been demonstrated that transgene-enforced overexpression of DRAK2 led to enhanced T cell apoptosis and a resultant decrease in memory T cell pools (43). While seemingly contradictory to the results presented here, we note that these mice were produced using a human β-actin promoter, a promoter likely to give rise to unregulated and ectopic expression of the DRAK2 transgene in a variety of cell types. It is likely that DRAK2 expression levels must be carefully regulated during T cell development and in naïve T cells, since this kinase plays significant roles in setting TCR signaling thresholds (40,60). As well, overexpression in these cells may trivially lead to apoptosis, as has been

observed in other cell types that do not normally express DRAK kinases (39,41,69). However, the basis for enhanced T cell apoptosis in such transgenic mice remains to be described.

While the direct targets of DRAK2's enzymatic activity are currently unknown, DRAK2 likely participates at some level in Ca^{2+} signaling in primary lymphocytes. *Drak2*−/− T cells and thymocytes mobilize Ca^{2+} to a much greater degree than wildtype T cells in response to weak TCR stimulation. Recently, we have observed that antigen receptor stimulation of T and B cells led to DRAK2 autophosphorylation of Ser12 (62). The antigen-receptor mediated induction of DRAK2 enzymatic activity is highly dependent upon Ca^{2+} mobilization, since calcium chelators blocked Ser12 autophosphorylation. Moreover, depletion of intracellular Ca^{2+} is sufficient to activate DRAK2 since treatment of lymphocytes with the SERCA pump inhibitor thapsigargin alone induces DRAK2 Ser12 autophosphorylation. These studies suggest that DRAK2 serves in a negative feedback loop to restrict levels of Ca^{2+} in lymphocytes responding to antigenic stimulation. Although DRAK2 lacks a calmodulin-binding motif, that Ca^{2+} modulates the activity of DRAK2 is not surprising given that other DAP kinase family members are also subject to its regulation.

Ca^{2+} has long been known to play significant roles in lymphocyte activation and survival (70). The recent discoveries of Stim1 and Orai1, the components of store-operated channels that allow heightened and sustained cytoplasmic Ca^{2+} following depletion of ER stores (71–74), have provided further impetus for investigating the exquisite control lymphocytes must exert over this fundamental second messenger. Importantly, Ca^{2+} controls the balance between life and death for many cell types, and its dysregulation under pathological states is often deleterious (75). In the context of T cells, Ca^{2+} signaling controls both the activation and survival status of responding clones. One example of this is the factor calcipressin-1 (Csp1), a negative regulator of calcineurin. Csp1 sets the threshold for NFAT activity in T cells via its restriction of calcineurin, and its deletion not only leads to enhanced T cell activation, but heightened expression of FasL and AICD hypersensitivity (25). The myelin basic protein splice variant golli also controls the activation and survival status of T cells *in vivo* by restraining store-depletion induced Ca^{2+} signaling in primary T cells (76). Similar to *Drak2*−/− T cells, *golli*−/− T cells are hyperproliferative to TCR crosslinking, produce enhanced levels of IL-2 upon suboptimal stimulation, and are refractory to $MOG_{35–55}$ induced EAE (77). While the basis for the enigmatic autoimmune resistance of *golli*−/− mice remains to be determined, it may be that T cells lacking golli are apoptotically hypersensitive due to defective Ca^{2+} homeostasis in an analogous fashion.

In our work, we have established that DRAK2 plays dual roles in T cells, first to restrict signal transduction downstream of the TCR and second, to maintain the survival of activated T cells. Since DRAK2 is an enzyme and may have multiple substrates in lymphocytes, it is quite possible that these are independent enzymatic functions. For example, naïve T cell activation may be restricted by one signaling cascade regulated by DRAK2, whereas blockade of apoptosis via the regulation of pro-survival factors like Bcl-xL may be orchestrated by a discrete pathway independently affected by DRAK2 activity. Alternatively, DRAK2 may affect these distinct features of T cell biology through the same signaling cascade. Studies are currently underway to carefully establish the targets of DRAK2 kinase activity to address this important question. Nevertheless, the studies presented here suggest that DRAK2 may be an important target for certain classes of autoimmune disease. While DRAK2 activity is necessary for the development of EAE, DRAK2 likely is involved in the development of other autoimmune diseases. This provokes an important question: why would a protein such as DRAK2 significantly impact autoimmune responses but only modestly participate in antimicrobial immunity? It is likely that DRAK2 plays other roles in controlling immunity. For example, DRAK2 function is essential for T cell help during germinal center reactions, and *Drak2*−/− mice fail to produce high affinity Abs; rescue of germinal center *Drak2*−/− T

cells with a Bcl-xL transgene led to a restoration of germinal centers and high affinity Abs (78). Further, *Drak2*−/− mice display defects in the maintenance of encephalitogenic West-Nile virus-reactive T cells, leading to resistance of these mice to lethal encephalitis (79). Despite this, *Drak2*−/− mice possess normal immune responsiveness to acute viral infection with LCMV and MHV (40,44). Thus, reagents to specifically block DRAK2 function may very likely have significant clinical utility. Most current therapies against autoimmune disease result in generalized immunosuppression. Given the results presented here, inhibition of DRAK2 may specifically prevent autoimmunity by targeting self-reactive T cells for clonal deletion without dramatically affecting the integrity of a normal immune response against acute microbial infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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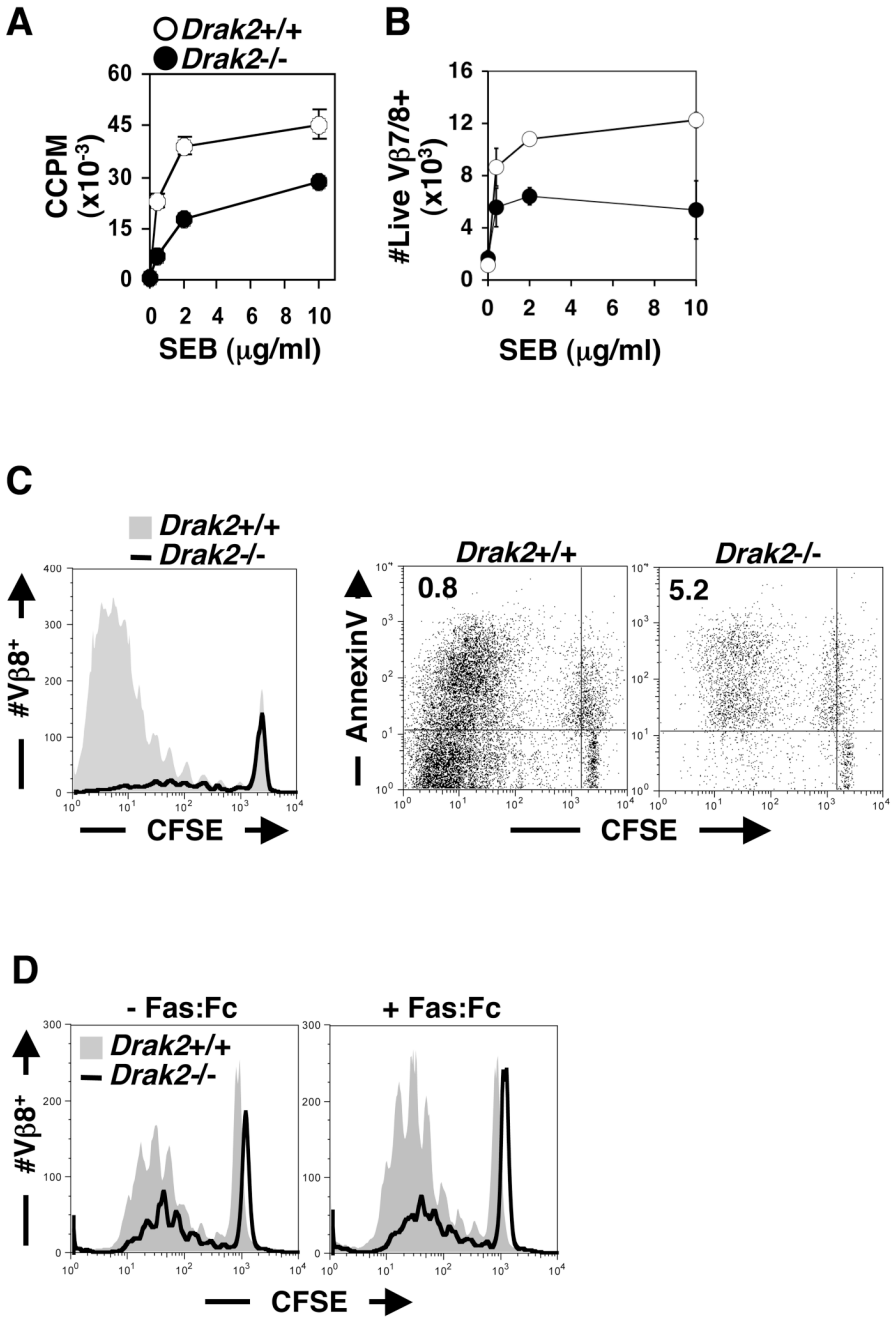

Figure 1. *Drak2*−/− T cells undergo abortive proliferation in response to superantigen

(a, b) Defective proliferation and live cell recovery of *Drak2*−/− T cells following stimulation with SEB. Purified T cells were stimulated with SEB in the presence of wildtype APCs, as described in the Experimental Procedures, and then assayed for (a) ^{3}H-thymidine incorporation in triplicate on day 3 or (b) Vβ7/8^{+} T cell recovery on day 8. Error bars indicate +/− standard deviation (S.D.). (c) Enhanced apoptosis in SEB-stimulated *Drak2*−/− T cells. CFSE-labeled purified T cells were stimulated with 2 μg/ml SEB for 8d then collected for FACS as previously described. Histogram (left panel) and dot plots (right panel) are gated on Vβ8^{+} T cells. Histogram (left) is based on live gate. Values indicate the ratio of dividing cells that are CFSE^{Lo}/annexin-V^{Hi} versus CFSE^{Lo}/annexin-V^{Lo} (right). (d) Enhanced apoptosis of SEB-

stimulated *Drak2*−/− T cells is independent of FasL signaling. Cells stimulated as in (c), but with Fas:Fc (1 μg/ml) added to cultures where indicated and analyzed after 4d. Data shown are representative of at least three independent experiments.

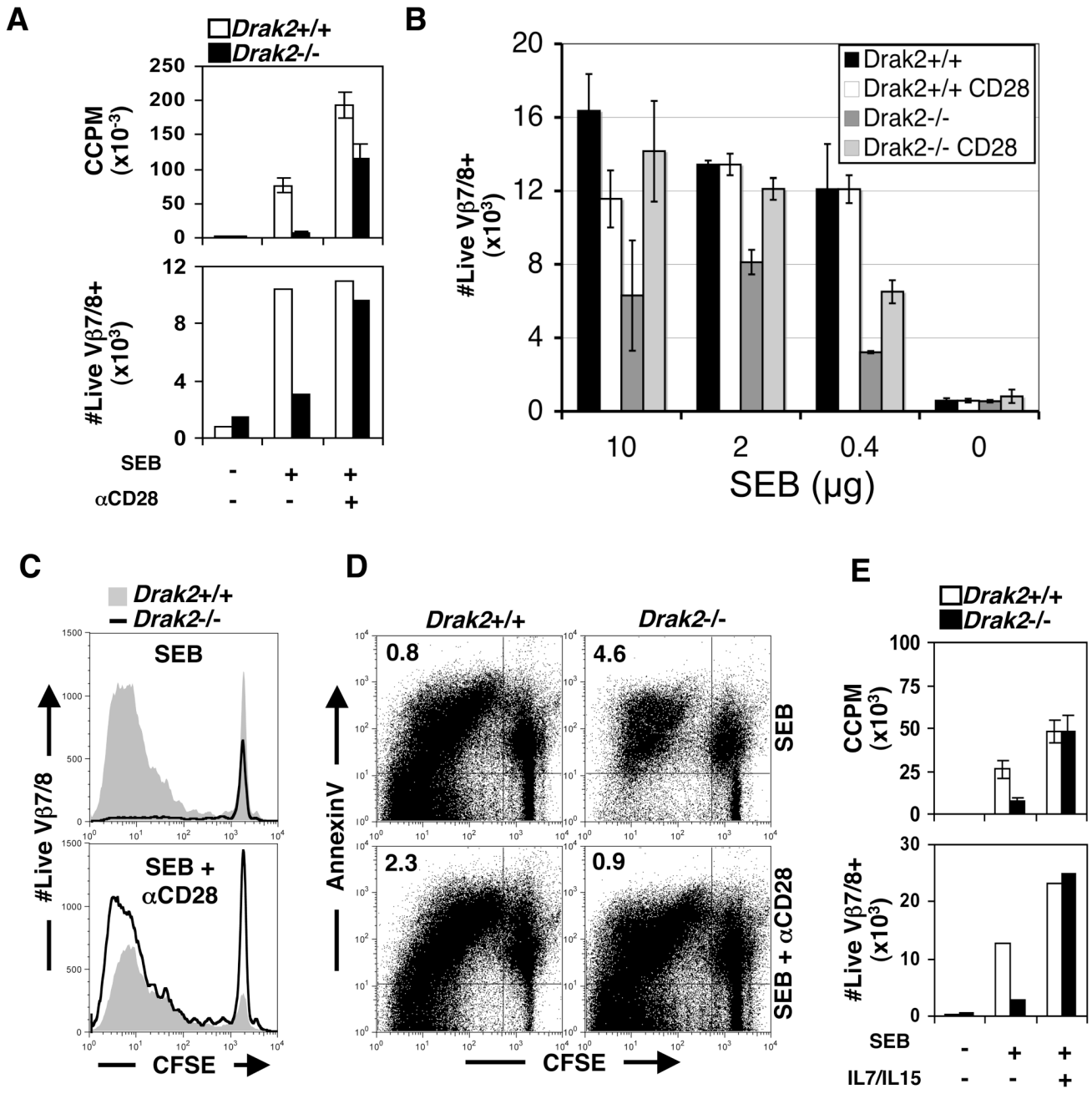

Figure 2. *Drak2*−/− T cell death to SEB is prevented by exogenous costimulation and homeostatic cytokines

Restoration of SEB-mediated clonal expansion of *Drak2*−/− T cells by anti-CD28. Purified T cells were activated with 2 μg/ml SEB with or without 500 ng/ml anti-CD28, then assayed as previously described for ^{3}H-Thymidine incorporation on day 6 (top) or Vβ7/8^{+} T cell recovery (bottom) on day 8. Error bars indicate standard deviation. (b) Anti-CD28 enhances recovery of *Drak2*−/− T cells stimulated with indicated doses of SEB, as in (a). Triplicate samples were incubated with the following doses of SEB pulsed onto APCs, with or without addition of 500 ng/ml anti-CD28, and collected after 8d culture followed by assessment of live Vβ7^{+} and Vβ8^{+} T cells as in (a). Error bars = +/−S.D. (c) Enhancement of the survival of proliferating

Drak2−/− T cells following SEB stimulation. CFSE-labeled purified T cells were stimulated with SEB for 8d with or without anti-CD28 (500 ng/ml) as indicated, and then analyzed by cytometry; live-gated/ Vβ7/8^{+} events plotted. (d) Anti-CD28 reduces apoptosis of proliferating *Drak2*−/− T cells following SEB-stimulation. Cells stimulated as in (c) were stained with Annexin-V, and total events were plotted. Dot plots gated on live Vβ7/8^{+} T cells; values indicate the ratio of dividing cells that are CFSE^{Lo}/annexin-V^{Hi} versus CFSE^{Lo}/annexin-V^{Lo}. (e) Rescue of SEB-stimulated Drak2−/− T cells by homeostatic cytokines IL-7 and IL-15. Cells were stimulated and analyzed as in (a); IL-7 and IL-15 (5 ng/ml each) were added to cultures where indicated. Results are representative of a minimum of three independent experiments conducted under identical conditions.

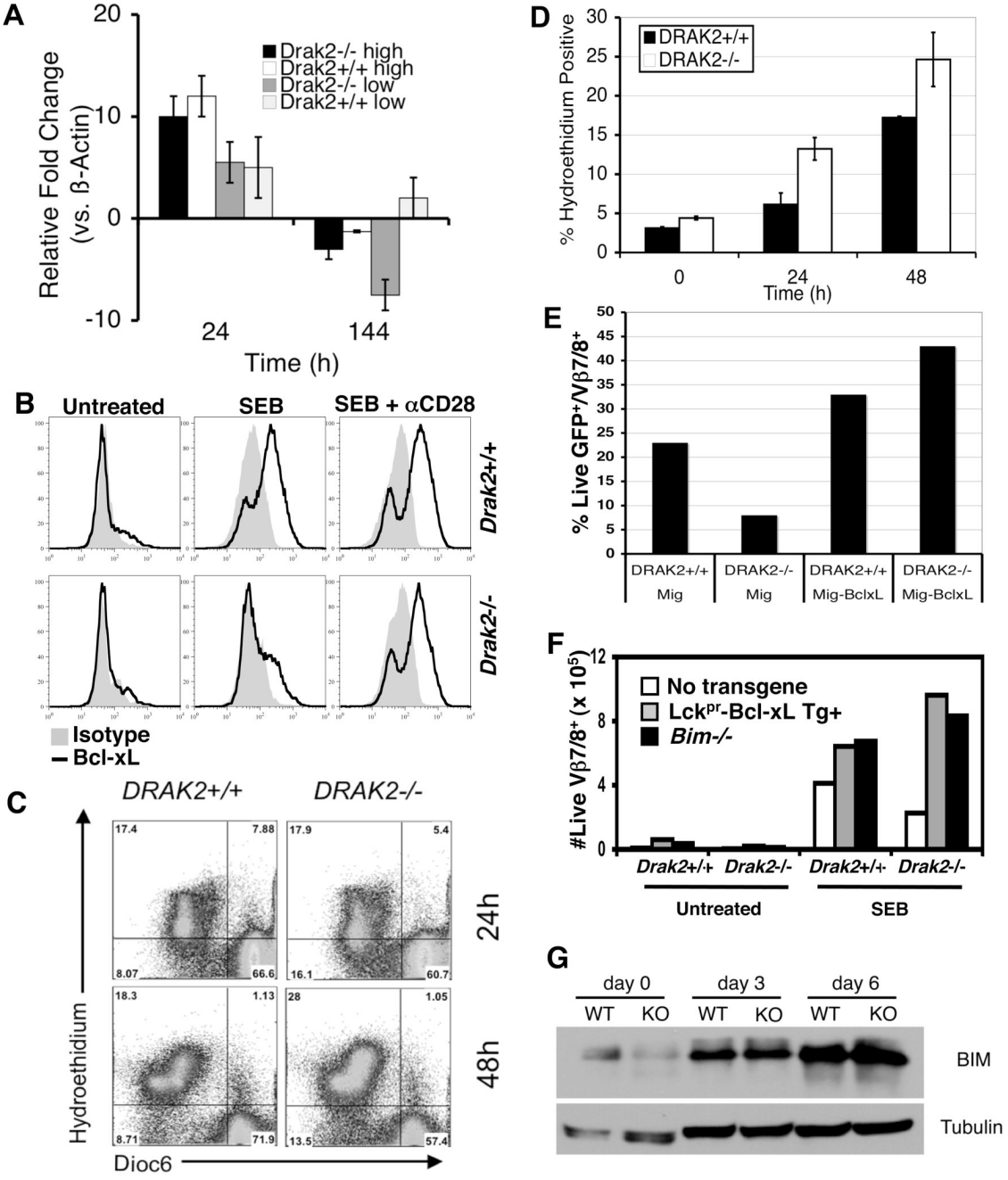

Figure 3. Bcl-xL expression restores *Drak2*−/− T cell survival in response to SEB

(a) Diminished maintenance of Bcl-xL expression in *Drak2*−/− T cells following mitogenic stimulation. Wildtype or *Drak2*−/− T cells were stimulated with plate-bound anti-CD3 (low = 100 ng/ml, high = 1000 ng/ml) without or with anti-CD28 (low = 0 ng/ml, high = 1 μg/ml) with some cultures harvested at 24h; after 48h, some cells were transferred to fresh wells with 100 U/ml IL-2, and harvested after 144h for quantitative realtime PCR as described in the Experimental Procedures. Data points represent the mean of triplicate samples +/− S.D., and were observed in two independent experiments (b) Defective upregulation of Bcl-xL in SEB-stimulated *Drak2*−/− T cells is restored by anti-CD28. T cells were activated with 2 μg/ml SEB plus wildtype splenic APCs with or without 500 ng/ml anti-CD28 and then analyzed on day 6

for Bcl-xL expression by intracellular staining, as described in the Experimental Procedures. Similar results were obtained in two independent experiments. (c, d) Enhanced loss of mitochondrial potential and reactive oxygen generation in mitogen-stimulated *Drak2*−/− T cells. Purified $CD4^+$ T cells of the indicated genotypes were stimulated with plate-bound anti-CD3 (200 ng/ml) plus soluble anti-CD28 (500 ng/ml) and harvested at 24 and 48h, followed by staining with DiOC6(3) to detect mitochondrial potential ($\Delta\psi_m$) and hydroethidium to indicate the presence of reactive oxygen species. The proportion of T cells staining for high levels of hydroxyethidium fluorescence are plotted; this was evaluated in triplicate and the mean +/− S.D. is plotted in (d). (e) Rescue of SEB-stimulated *Drak2*−/− T cells by retroviral expression of Bcl-xL. T cells were infected with the indicated MSCV-based retroviruses at 48 hours and 72 hours following activation with SEB; MIG contains an IRES-GFP, allowing a determination of recovery of infected cells. 18 hours later retroviral supernatant was replaced with fresh media and cells cultured for a total of 8 days and then collected for cytometric analysis. The percent of GPF^+/live $V\beta7/8^+$ was determined for cells infected with empty MiG retrovirus vs. MiG-BclxL retrovirus. Similar results from an independent experiment are provided in Supp. Fig. 4. (f) Purified T cells from mice of the indicated genotypes (*Drak2*+/+, *Drak2*−/−, Lck^{pr}-Bcl-xL Tg, *Drak2*−/− x Lck^{pr}-Bcl-xL Tg, *Bim*−/−, or *Drak2*−/− x *Bim*−/−) were stimulated with 2 μg/ml SEB pulsed wildtype splenic APCs and cultured for 8d, followed by determination of GFP-expressing $V\beta7/8^+$ live cell recovery. (g) Bim expression is not impacted by a DRAK2 deficiency in T cells following SEB stimulation. Wildtype (WT) and *Drak2*−/− (KO) T cells were incubated with wildtype APCs pulsed with 2 μg/ml SEB, and samples were collected at the indicated times, followed by Ficoll purification and magnetic enrichment for Vβ7 and Vβ8 TCR expression; similar results were obtained in an identical experiment (not shown). Unless otherwise noted, results presented are representative of three independent experiments conducted using identical conditions.

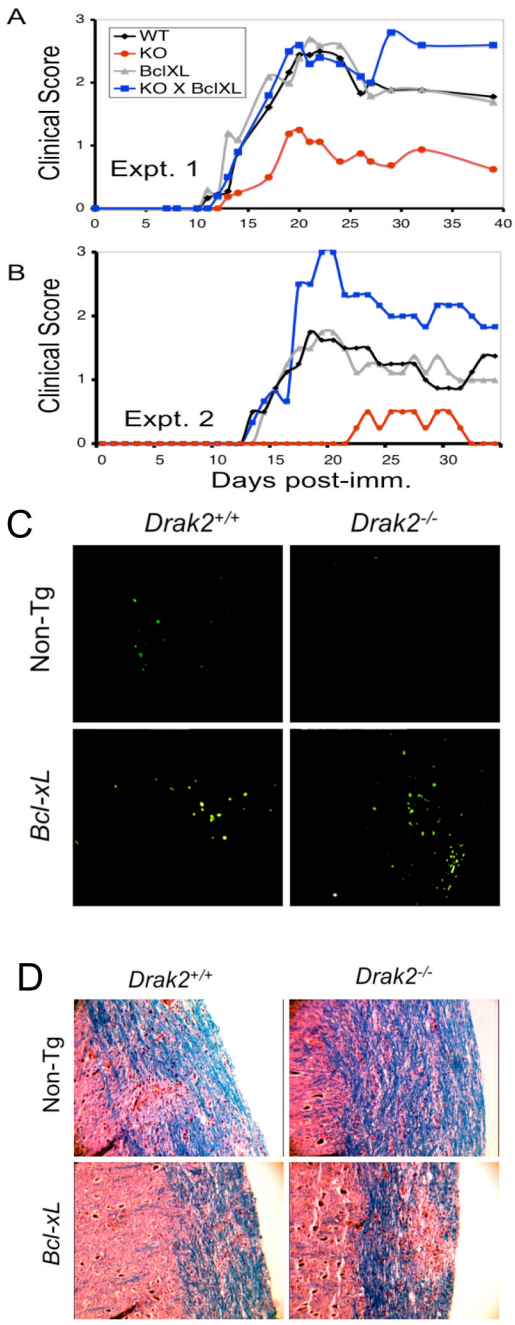

Figure 4. Bcl-xL expression restores susceptibility of *Drak2*−/− mice to EAE

(a and b) EAE sensitivity is restored in *Drak2*−/− mice by a T cell intrinsic Bcl-xL transgene; two independent experiments shown. *Drak2*+/+ (n=15), *Drak2*−/− (n=15), Lck^{pr}-Bcl-xL Tg (n=5), or *Drak2*−/− Lck^{pr}-Bcl-xL Tg (n=5) mice received 250μg $MOG_{35–55}$ peptide plus CFA on days 0 and 7 and 200 ng pertussis toxin on days 0, 2, and 7. Mice were scored as follows: (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 fore limb paralysis, (5) death. * $p<0.05$ for *Drak2*+/+ vs. *Drak2*−/− mice. ^ $p<0.05$ for Lck^{pr}-Bcl-xL Tg vs. *Drak2*−/− Lck^{pr}-Bcl-xL Tg mice. (c) T cell recruitment into the CNS of *Drak2*−/− is restored by a Bcl-xL transgene. Mice of the indicated genotype were immunized with $MOG_{35–55}$ as in (a

and b). Brain sections were prepared after 25d immunization and stained with anti-CD3-FITC. (d) Increased demyelination in spinal chords of *Drak2*−/− x *Bcl*-xL mice. Sections of spinal cords recovered from mice undergoing $MOG_{35–55}$ challenge were harvested at 25d and stained with luxol fast blue to detect myelin stripping, as described in the Experimental Procedures. Similar results were obtained in two independent studies.

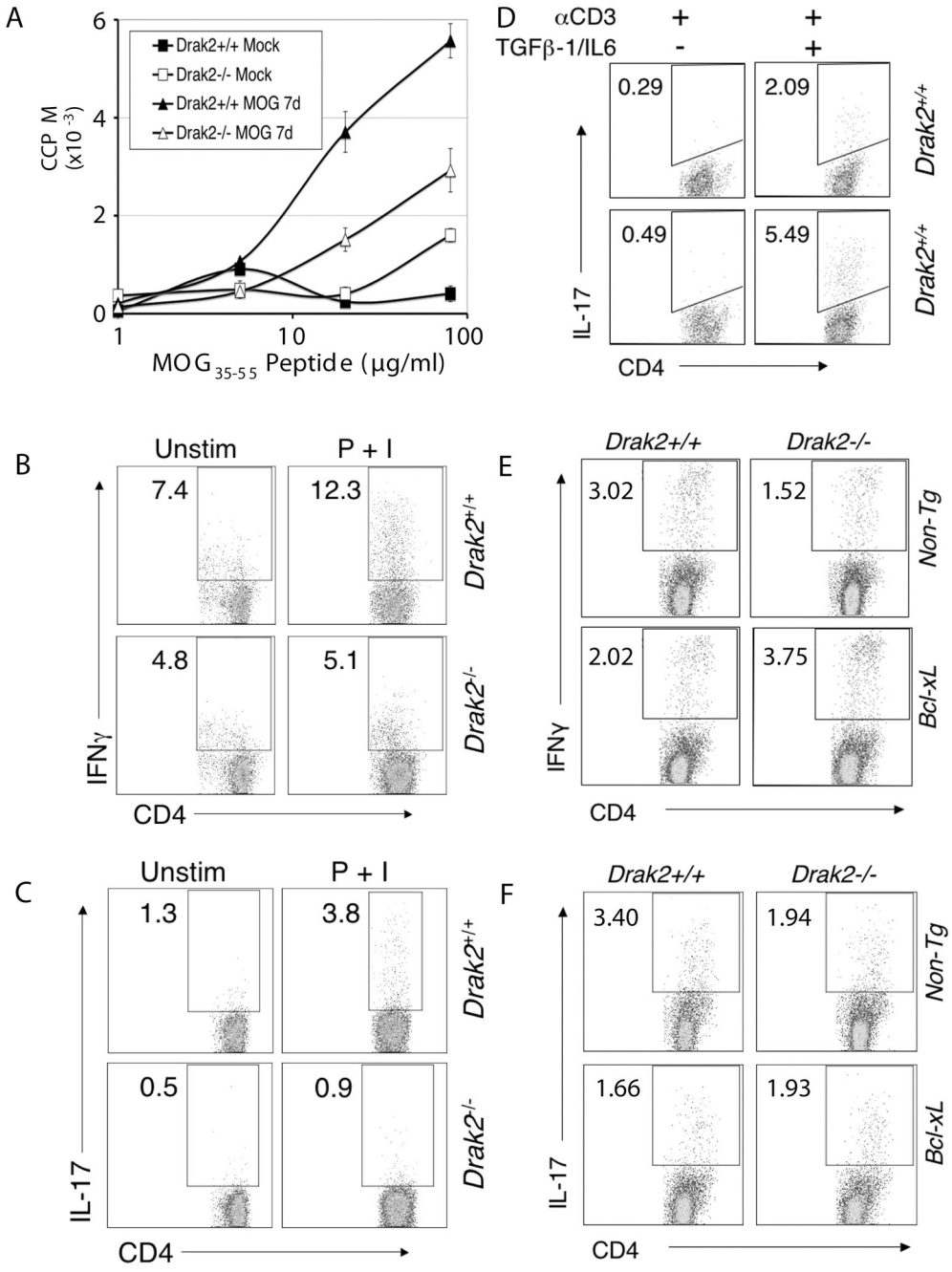

Figure 5. Defective maintenance of Th1 and Th17 cells in $MOG_{35–55}$ challenged *Drak2*−/− mice

Diminished recovery of $MOG_{35–55}$ specific *Drak2*−/− T cells after 7d *in vivo* challenge. Mice of the indicated genotype (n=3) were immunized with $MOG_{35–55}$ plus CFA as described in the Experimental Procedures. Cells were harvested at 7d, and purified $CD4^+$ T cells were incubated in triplicate with wildtype splenic APCs coated with the indicated concentration of $MOG_{35–55}$ peptides. After 4d *ex vivo* culture, ^{3}H-thymidine was added for the final 18h followed by harvesting and scintillation counting. Mean values +/− S.D. shown. *Drak2*−/− fail to maintain Th1 (b) and Th17 (c) T cells following $MOG_{35–55}$ challenge. Wildtype and *Drak2* −/− mice were challenged *in vivo* for 14d with $MOG_{35–55}$ as in (a), followed by *ex vivo* restimulation of purified $CD4^+$ T cells with PMA plus ionomycin (P + I), or left unstimulated

(Unstim) for 6h. Cells were stained with anti-CD4-APC, fixed, permeabilized and then stained with anti-IFNγ-PE (b) or anti-IL-17-PE (c). Values are representative of three independent experiments and represent the proportion of $IFN\gamma^+$ or $IL\text{-}17^+$ $CD4^+$ T cells in (b) and (c), respectively. (d) Th17 differentiation under biasing conditions is not impaired by a DRAK2 deficiency. T cells of the indicated genotype were incubated with a five-fold excess of wildtype APCs, anti-CD3 (2 μg/ml) with or without TGFβ-1 (10 ng/ml) plus IL-6 (20 ng/ml). After 4d, cells were re-plated with 100 U/ml IL-2. After 6d, cells were incubated with PMA (40 ng/ml) plus ionomycin (1 μg/ml), and treated for intracellular staining as described in the Methods section. The proportion of CD4-gated IL-17 expressing cells is shown. (e, f) Restoration of MOG-reactive Th1 clones by a Bcl-xL transgene. Wildtype (*Drak2*+/+) and *Drak2*−/− mice, with (Bcl-xL) or without (Non-Tg) a Bcl-xL transgene, were immunized with MOG35–55, as described in the Methods section. After 14d, T cells were harvested and purified. These were added to MOG35–55 pulsed APCs for 24h, followed by staining for CD4, permeabilization, and subsequent staining for IFNγ (e) and IL-17 (f). Results are representative of at least three independent experiments.

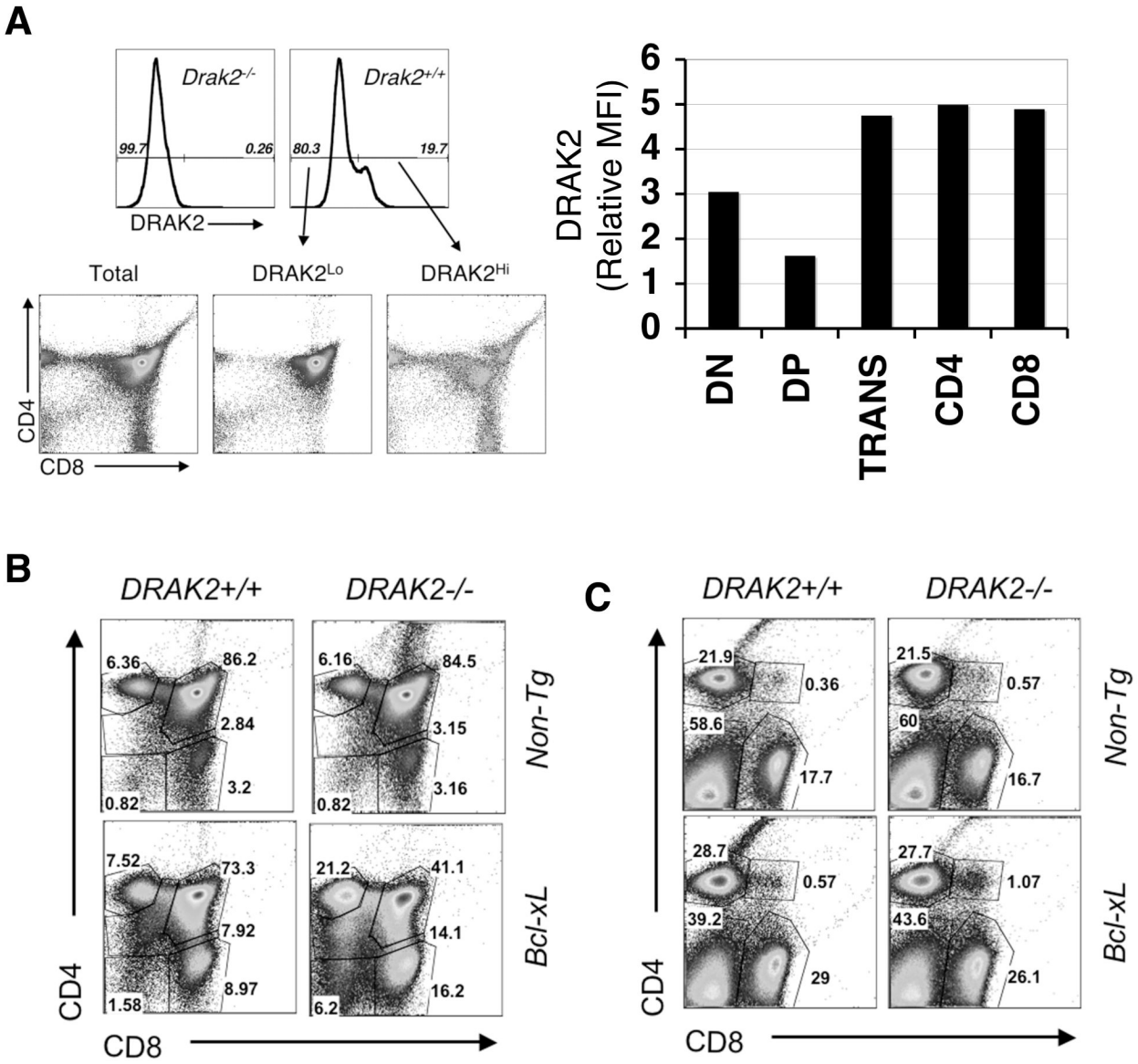

Figure 6. Enhanced positive selection of *Drak2*−/− thymocytes revealed by a Bcl-xL transgene

(a) High level DRAK2 expression in thymocytes undergoing selection. Thymocytes from wildtype and *Drak2*−/− were stained with anti-CD4 and anti-CD8, followed by fixation, permeabilized, staining with anti-DRAK2, and FITC-conjugated anti-rabbit Abs. As a control, wildtype thymocytes were also stained with an isotype control Ab (not shown). Wildtype thymocytes were gated for DRAK2 expression (top left panel) and CD4 vs. CD8 dotplots shown for all cells, DRAK2 low and DRAK2 high staining cells (bottom left panel). MFI values for anti-DRAK2 mAb staining were obtained and the ratio of MFIs for wildtype vs. *Drak2*−/− T cell control staining is shown (right panel); isotype controls demonstrated a staining pattern similar to Drak2−/− samples (not shown). Similar results were obtained in a separate experiment (b,c) CD4 vs. CD8 phenotypes of thymic (b) and splenic (c) T cell subsets from *Drak2*+/+, *Drak2*−/−, *Bcl-xL*, and *Drak2*−/− *x Bcl-xL* mice. Thymocytes and splenocytes were harvested from female littermates of the indicated genotypes at 8wk, stained with anti-CD4 and anti-CD8 and analyzed by flow cytometry. Representative dot-plots of five independent experiments are provided.