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DRAK2 regulates memory T cell responses following murine coronavirus infection

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Abstract
The contribution of DRAK2 [death-associated protein kinase (DAPK)-related apoptosis-inducing kinase 2] to anti-viral memory T cell responses following infection with mouse hepatitis virus (MHV) was examined. DRAK2 is a lymphoid-enriched serine/threonine kinase that is an important regulatory molecule involved in modulating T cell responses. Memory T cells derived from MHV-immunized *Drak2* $^{-/-}$ mice exhibited amplified proliferation and IFN-γ secretion following stimulation with viral epitopes. Transfer of *Drak2* $^{-/-}$ memory T cells into *Rag1* $^{-/-}$ mice infected intracerebrally with MHV resulted in accelerated clearance of virus from the brain. Thus, DRAK2 may be a novel target for stimulating protective immunity to viral pathogens.

Keywords: *DRAK2, virus, T cells, memory, host-defense*

DRAK2 [death-associated protein kinase (DAPK)-related apoptosis-inducing kinase 2] is a lymphoid-enriched serine/threonine kinase that has emerged as an important regulatory molecule involved in modulating T cell responses [1–4]. While DRAK2 possesses homology with members of the DAPK family, kinases that potentiate apoptotic signaling [5], DRAK2 does not appear to function as a pro-apoptotic effector in thymocytes or peripheral T cells [1,2]. Rather, T cells from *Drak2*-deficient mice maintain a lower threshold for T cell receptor (TCR)-mediated stimulation and hyperproliferate in response to suboptimal costimulatory signals [2]. Since DRAK2 kinase activity is both induced and inhibitory toward TCR signaling, current evidence suggests that DRAK2 signaling constitutes a negative feedback loop during T cell activation [4]. In support of this, mice bearing a homozygous deletion of the *Drak2* gene (*Drak2* $^{-/-}$ mice) do not develop spontaneous autoimmune disease and are resistant to myelin oligodendrocyte (MOG) peptide-induced experimental autoimmune encephalomyelitis (EAE) [2,3].

Mouse hepatitis virus (MHV) is a member of the *Coronaviridae* family that can infect the central nervous system (CNS) resulting in an acute encephalomyelitis [6–8]. MHV-specific $CD4^+$ and $CD8^+$T cells are critical for elimination of replicating virus from the CNS [9–13] by secretion of IFN-γ and cytolytic activity. We have recently determined that *Drak2*-deficient mice are able to effectively reduce viral burden within the CNS following MHV infection, demonstrating that both anti-viral T cell function and trafficking of effector T cells generated during acute disease is not impaired in the absence of DRAK2 signaling [3]. The present study was undertaken to assess the role of DRAK2 signaling in anti-viral memory T cell responses.

Drak2 $^{+/+}$ (C57BL/6, H-2^b background, National Cancer Institute, Bethseda, MD) and *Drak2* $^{-/-}$ mice (*Stk17* ^{bm1Hed}; C57BL/6, H-2^b) [2,3] were infected intraperitoneally (i.p.) with 2×10^5 PFU of MHV-DM [14] and memory T cells within spleens were evaluated at day 33 post-infection (p.i.). Comparable numbers of T cells bearing the $CD44^{hi}/CD62^{lo}$ memory phenotype

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were present within infected *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ mice as determined by flow cytometry using previously described methods [15] (data not shown). Intracellular staining for IFN-γ in splenocytes pulsed with the immunodominant CD4 epitope present within the membrane (M) glycoprotein at residues 133–147 (M133–147) [16] revealed a small increase ($p < 0.05$) in the frequency of epitope-specific CD4 + T cells in *Drak2* $^{-/-}$ mice (Figure 1(A)), which translated to similar overall numbers compared to *Drak2* $^{+/+}$ mice (Figure 1(B)). There were no significant differences between the frequency and number of virus-specific CD8 + T cells recognizing the immunodominant CD8 epitope located within residues 510–518 of the MHV spike (S) glycoprotein (S510–518) as determined using the PE-conjugated D^b/S510–518 major histocompatibility class I tetramer [3] (Figure 1(A),(B)). These data indicate that DRAK2 signaling does not dramatically influence the generation of virus-specific memory T cells.

We next tested if virus-specific T cells from *Drak2*-deficient mice displayed increased IFN-γ production following antigenic challenge. Splenocytes were isolated from *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ mice at day 33 p.i. and stimulated *in vitro* for 48 h with a range of M133–147 and S510–518 (0.05–5.0 μM) peptide or OVA peptide as a negative control. Supernatants were harvested and IFN-γ was quantified using the Mouse IFN-γ DuoSet (R&D Systems, Minneapolis, MN). Enhanced IFN-γ secretion by memory $CD4^+$ (Figure 2(A)) and $CD8^+$ (Figure 2(B)) T cells isolated from MHV-immunized *Drak2* $^{-/-}$ mice was observed at all concentrations tested compared to *Drak2* $^{+/+}$ mice. Further, memory T cells from *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ cells displayed a differential ability to react to low concentrations of M133–147 and S510–518 indicating that the absence of DRAK2 lowers the threshold for activation in memory T cells following viral infection as determined by IFN-γ secretion.

Memory T cells from *Drak2* $^{-/-}$ mice also exhibited enhanced proliferation following re-exposure to viral antigen. Splenocytes were isolated from *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ mice at day 33 p.i. and cultured in the presence of 0.05, 0.5 or 5.0 μM of either M133–147, S510–518 or OVA peptide for 5 days. Cells were stimulated for 4 days with M133–147 or S510–518, pulsed with 1 μCi/ml [^{3}H]-thymidine (NEN Research Products, Boston MA) for 24-hours, harvested and [^{3}H] incorporation was quantified using a scintillation counter. Hyperproliferation of $CD4^+$T cells was observed in *Drak2*-deficient mice compared to wild-type indicated by ~40–70% increase in the amount of [^{3}H]-labeled thymidine incorporation (Figure 3(A)). *Drak2* $^{-/-}$ $CD8^+$T cells also divided more rapidly following S510–518 stimulation at all concentrations tested (Figure 3(B)). By contrast, negative control OVA peptide did not induce significant levels of thymidine incorporation in cells from *Drak2* $^{+/+}$ or *Drak2* $^{-/-}$ mice (Figure 3(A),(B)). Thus, DRAK2 appears to play an important role in regulating cell proliferation after re-challenge with viral antigen. Further, these results suggest that the elevated level of IFN-γ produced from *Drak2* $^{-/-}$ splenocytes was influenced by the overall increase in the total number of *Drak2* $^{-/-}$ virus-specific memory T cells following hyperproliferation.

The amplified anti-viral response in memory T cells in *Drak2* $^{-/-}$ mice correlated with accelerated reduction of viral titers within infected tissue. Splenocytes derived from MHV-immunized *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ mice were adoptively transferred via intravenous injection

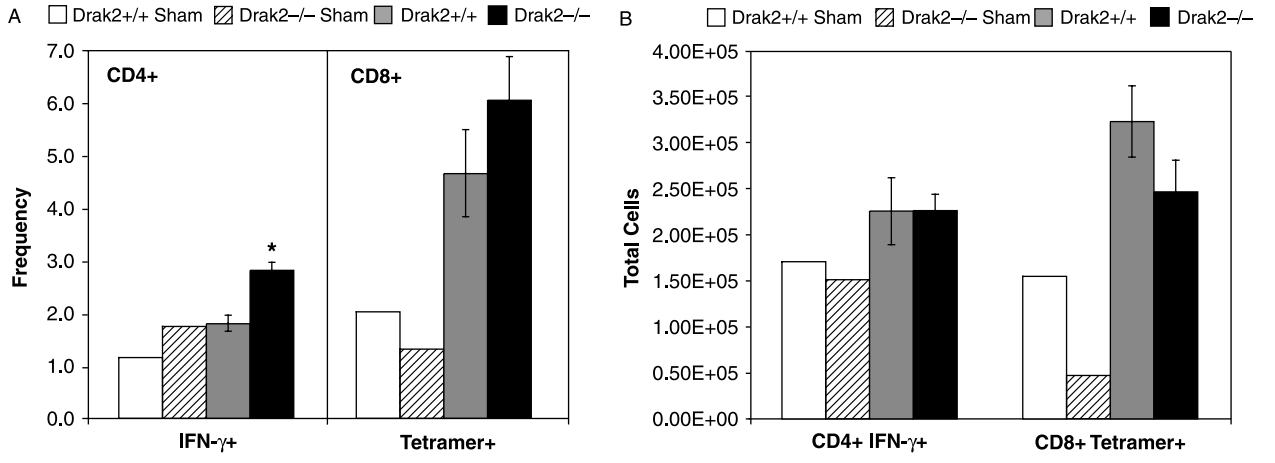

Figure 1. Memory T cell generation in *Drak2*-deficient mice. The frequencies (A) and total numbers (B) of virus-specific $CD4^+$ and $CD8^+$T cells were observed in MHV-infected wild-type (*Drak2* + /+) and *Drak2*-deficient (*Drak2* − /−) mice and mock-infected wild-type (*Drak2* + /+ Sham) and knock-out (*Drak2* − /− Sham) mice, as determined by intracellular IFN-γ and tetramer staining respectively. The data presented in (A) and (B) are the average ± SEM. Independent experiments were performed a minimum of 3 times with 5 mice per group. An asterisk above the bar represents a statistically significant difference ($p \leq 0.05$) between *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ mice calculated by the Mann–Whitney test.

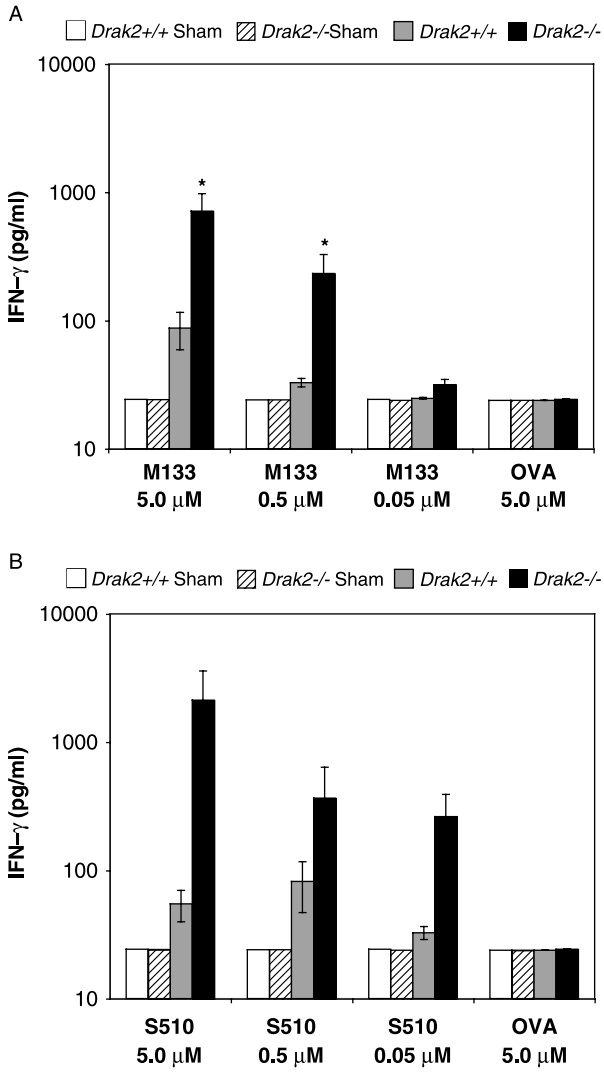

Figure 2. IFN-γ production in *Drak2*-deficient memory T cells in response to viral antigen. IFN-γ production was amplified in $CD4^+$T cells from *Drak2*-deficient (*Drak2* − /−) mice immunized with MHV compared to wild-type (*Drak2* + /+) over all concentrations of M133–147 tested (A). Similarly, stimulation with S510–518 also enhanced the IFN-γ response in *Drak2*-deficient CD8 + T cells (B). In contrast, no difference in IFN-γ production was noted in MHV-infected $Drak2^{+/+}$ or $Drak2^{-/-}$ cells compared to those from mock-infected wild-type (*Drak2* + /+ Sham) and knock-out (*Drak2* − /− Sham) mice following stimulation with 5.0 μM OVA peptide as a negative control (A, B). Data presented in (A) and (B) are the average ± SEM. Independent experiments were performed 2 times with a minimum of 3 mice per group. An asterisk above the bar represents a statistically significant difference ($p \leq 0.05$) between $Drak2^{+/+}$ and $Drak2^{-/-}$ mice calculated by the Student's *t* test.

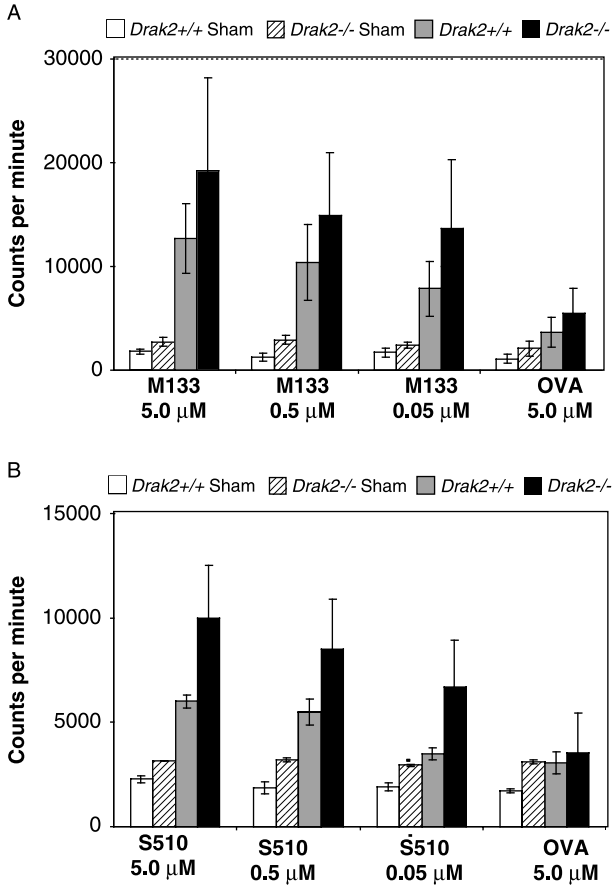

Figure 3. Proliferation of *Drak2*-deficient T cells following antigenic stimulation. (A) Splenocytes from MHV-infected Drak2-deficient (Drak2 − /−) mice stimulated with M133–147 showed increased replication compared to wild-type (*Drak2* + /+) indicated by an overall increase in the counts per minute of $[^3H]$. (B) Stimulation with S510–518 also resulted in hyperproliferation of $Drak2^{-/-}$ cells. In contrast, there was not a significant proliferative response to viral antigens in splenocytes from mock-infected wild-type (*Drak2* + /+ Sham) or knock-out (*Drak2* − /− Sham) mice. Data presented in (A) and (B) are the average ± SEM. Independent experiments were performed four times with 5 mice per group.

(2.5×10^6 total cells) into $Rag1^{-/-}$ mice (C57BL/6, H-2^b background, National Cancer Institute) that had been infected intracerebrally with 500 PFU of the neurotropic MHV strain J2.2 3 days prior to transfer [17]. Importantly, similar numbers of donor $CD4^+$ and $CD8^+$T cells specific for either the M133–147 or S510–518 epitopes respectively, were injected into $Rag1^{-/-}$ recipients. Mice were sacrificed between 5–8 days post-transfer and brains were collected to determine T cell accumulation and viral titer using previously described methods [18,19]. An ~2-fold increase in the frequency and total number of $CD4^+$ and $CD8^+$T cells were present within the brains of $Rag1^{-/-}$ mice receiving $Drak2^{-/-}$ cells compared to wild-type recipients (Figure 4(A),(B)). Tetramer staining showed a modest increase in the frequency of virus-specific $CD8^+$T cells from $Drak2^{-/-}$ (62% ± 2.6, $n = 3$) compared to $Drak2^{+/+}$(54% ± 1.7, $n = 3$) mice (Figure 4(C)). Further analysis confirmed that there was roughly a 2-fold increase in total $Drak2^{-/-}$ virus-specific $CD8^+$T cells within the $Rag1^{-/-}$ brains compared to wild-type (Figure 4(D)). In addition, splenocytes derived from $Drak2^{-/-}$ mice controlled replicating virus more effectively than those from $Drak2^{+/+}$ mice demonstrated by a significant ($p \leq 0.05$) decrease in the viral titer within the brains

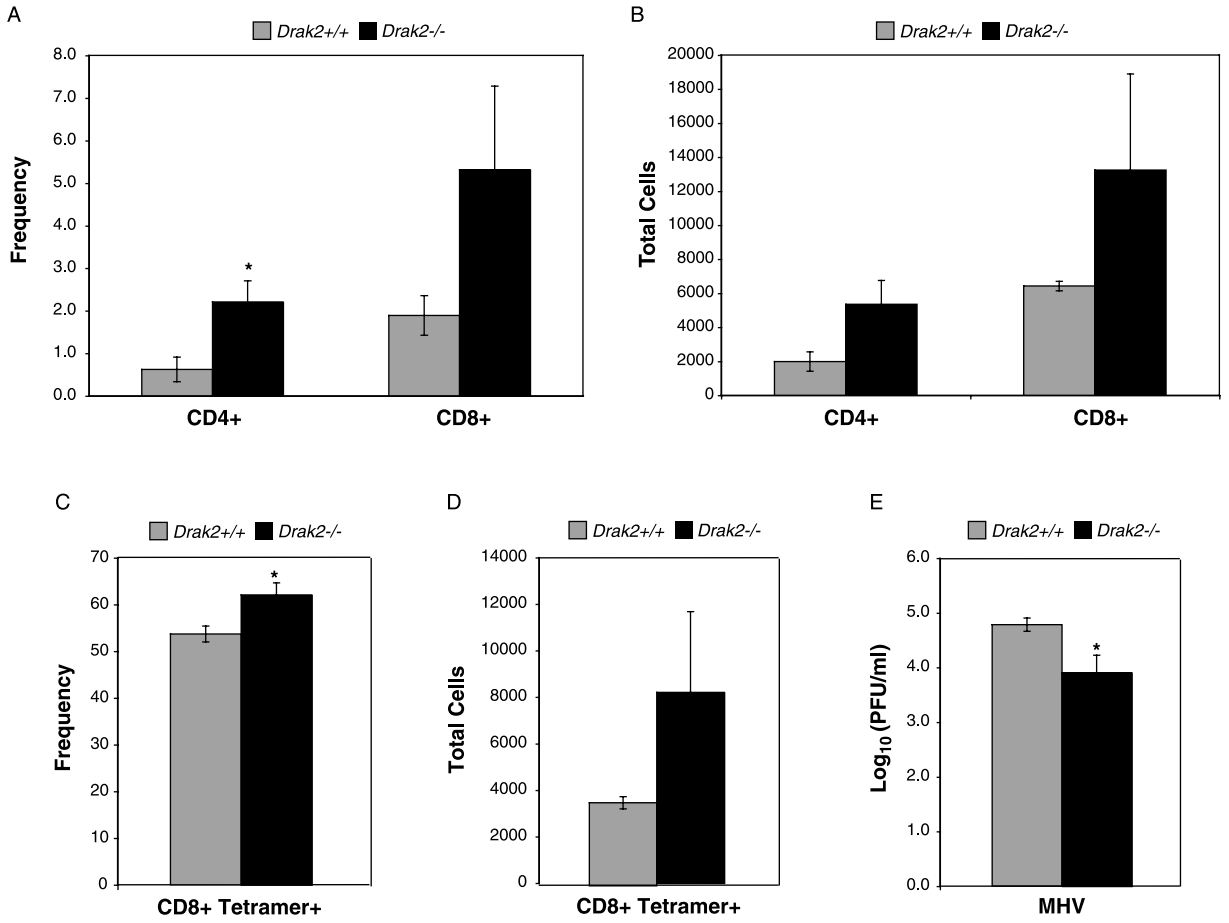

Figure 4. *Drak2* $^{-/-}$ T cell mediated viral clearance within the CNS of immunodeficient mice. $CD4^+$ and $CD8^+$T cells from *Drak2*-deficient (*Drak2* − /−) mice immunized with MHV accumulated to higher frequencies (A) and total numbers (B) within the brains of MHV-infected *Rag1* $^{-/-}$ mice compared to those receiving cells from wild-type (*Drak2* + /+) mice. The frequency (C) and total number (D) of $CD8^+$ virus-specific T cells was also increased in *Rag1* $^{-/-}$ mice that received *Drak2* $^{-/-}$ cells. *Drak2* $^{-/-}$ cells adoptively transferred to MHV-infected *Rag1* $^{-/-}$ mice were also capable of clearing replicating virus at a faster rate than wild-type cells (E). The data in (A)–(D) is the average ± SEM from one experiment with an $n = 3$ and is representative of 3 independent experiments performed with a minimum of 3 mice per group. An asterisk above the bar represents a statistically significant difference between *Drak2* $^{+/+}$ and *Drak2* $^{-/-}$ mice calculated by the Student's *t* test.

of *Rag1* $^{-/-}$ recipient mice (Figure 4(E)). Together, these data demonstrate that *Drak2* $^{-/-}$ T cells exert enhanced effector function following antigenic challenge *in vivo*.

Earlier studies revealed that DRAK2 signaling is not essential for T cell effector function following initial encounter with viral pathogens during acute disease [2,3], despite the observation that *Drak2* $^{-/-}$ T cells are hypersensitive to TCR stimulation [1,2]. This study demonstrates for the first time that *Drak2*-deficiency substantially increases the strength of the anti-viral recall response elicited by memory T cells. Virus-specific *Drak2* $^{-/-}$ memory T cells had a lower threshold for activation and increased proliferation following antigenic stimulation *ex vivo* that correlated with elevated IFN-γ levels when compared to wild-type mice. In addition, the *in vitro* findings correlated with accelerated reduction in viral burden within the brain following adoptive transfer of memory *Drak2* $^{-/-}$ T cells into immunodeficient mice infected with MHV. We attribute the increase in virus-specific *Drak2* $^{-/-}$ memory T cell numbers within the CNS to amplified T cell proliferation following re-exposure to antigen and not to enhanced trafficking as we have previously demonstrated that *Drak2*-deficiency does not influence T cell migration [3]. In light of our present findings, it appears the absence of DRAK2 signaling differentially influences the magnitude of anti-viral effector function, which is dictated by the stage of T cell activation, e.g. acute vs. memory.

The heightened response of *Drak2* $^{-/-}$ memory T cells following challenge with MHV may be related to physiologic changes that promote the quantity and/or quality of virus-specific T cells [20]. For instance, antigen-specific memory T cells are present at a higher frequency in immunized compared to naïve mice and this contributes to the anti-viral recall response [21–24]. We have found DRAK2 activity is induced

by TCR stimulation in naïve and effector T cells and that its catalytic activity is important for attenuating TCR signaling, implicating the kinase in a negative feedback loop [4]. Therefore, hypersensitivity of *Drak2* $^{-/-}$ T cells to TCR stimulation could also promote increased numbers of antigen-specific memory T cells compared to wild-type cells. However, our present data in conjunction with the LCMV study [2] suggest that DRAK2 does not dramatically influence overall numbers of virus-specific memory T cells following MHV infection. Alternatively, disruption of DRAK2 signaling may enhance the effect of other downstream pathways that amplify the quality of the T cell recall response. Accelerated effector function of memory T cells is associated with modifications of the gene expression profile through epigenetic changes, including reorganization of chromatin structure, DNA methylation and histone modification [25,26], leading to upregulation of active transcription factors [27] and constitutive cytokine gene expression [28–30]. These intracellular changes are reflected in the reprogramming of virus-specific T cells that contributes to the heightened anti-viral recall response of memory T cells [20]. It is of interest that transgenic mice designed to ectopically express DRAK2 have a diminished population of memory T cells, perhaps a consequence of enhanced apoptosis noted in these transgenic T cells [31]. These findings are consistent with previous reports demonstrating that DRAK2 hyperexpression leads to apoptosis [32,33]. However, the converse is not true; *Drak2* $^{-/-}$ T cells are not resistant to apoptosis. Rather, the enhanced memory observed here is likely the result of enhanced memory cell function on a per cell basis rather than due an enlarged memory pool. Thus, we propose that *Drak2* $^{-/-}$ mice exhibit an amplified anti-viral recall response relative to wild-type as a consequence of augmented TCR signaling in memory T cells.

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