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Loss of DRAK2 signaling enhances allogeneic transplant survival by limiting effector and memory T cell responses*

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Abstract

Here we demonstrate that loss of DRAK2 signaling significantly promotes the acceptance of allogeneic engraftment in two separate transplant models without promoting generalized immunosuppression. *Drak2*-/- T cells failed to reject allogeneic tumors, and were defective in rejecting Balb/C allogeneic skin grafts on C57BL6/J recipients. A significant fraction of alloreactive *Drak2*-/- T cells underwent apoptosis following activation, whereas enforced expression of *Bcl-xL* in *Drak2-/-* T cells restored allograft rejection. Formation of allogeneic memory was also greatly hampered in T cells lacking the *Drak2* gene. Adoptive transfer of memory T cells from *Drak2-/-* mice failed to promote the rejection of allogeneic tumors, and such cells led to significantly delayed rejection of skin allografts in the Balb/C->C57BL/6J model. Costimulatory blockade by *in vivo* administration of Cytotoxic T-Lymphocyte Antigen 4 fusion protein (CTLA4-Ig) synergized with the DRAK2 deficiency and led to long-term allogeneic skin graft acceptance. Overall, these results demonstrate that DRAK2 plays an important role in primary and memory T cell responsiveness to allografts. Since previous studies have demonstrated that a loss of DRAK2 does not negatively impact antiviral immunity, the studies here underscore the potential utility of pharmacological blockade of DRAK2 to achieve transplant maintenance without the imposition of generalized immunosuppression.

Keywords

Allograft; tolerance; kinase; costimulation; immunosuppression

Introduction

Following transplantation of an organ, tissue, or cell population from a foreign donor, recognition of polymorphisms within MHC proteins, termed allorecognition, typically leads to T cell activation and destruction of the allograft (1). Currently, allograft rejection can be prevented clinically through lifelong administration of powerful immunosuppressants such as cyclosporine A or FK506, but in many instances these drugs have attendant toxicity issues (2). Even with potent immunosuppression, allogeneic memory T cell (Tmem) responses have proven refractory to current treatment regimens, and organ failure often

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Supporting Information
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results from chronic rejection mediated by allogeneic Tmem (3). Therefore, allograft recipients must live in a chronically immunosuppressed state, with the risk that a deadly opportunistic infection or chronic rejection may occur (2).

At present, there are few known intracellular or extracellular targets that may be exploited to specifically block allograft rejection without imposing generalized immunosuppression. Blockade of T cell costimulatory ligands such as CD80/86 and CD40 have shown promise for preventing allograft rejection in mice, especially when these treatments are used in combination. CD40 blockade has been removed from clinical trials due to thromboembolic complications following administration, though CTLA4-Ig therapy is now approved for blocking certain alloresponses. (5). Exploration of additional therapeutic targets to prevent rejection without detrimental effects on recipient health remains imperative.

DRAK2 is a negative regulator of T cell activation, and is highly enriched in T and B cells (6). When DRAK2 expression is abolished through genetic deletion, mice develop resistance to the organ specific T cell mediated autoimmune diseases Experimental Autoimmune Encephalomyelitis (EAE), and are resistant to type-I diabetes that spontaneously develops in the non-obese diabetic mouse background (6-8). While T cells deficient in *Drak2* are more readily activated with suboptimal stimulation through the TCR, they die from apoptosis prior to accumulation (7). Importantly, previous studies have shown that loss of DRAK2 signaling does not impair anti-viral immunity to Murine Hepatitis Virus (MHV), Lymphocytic Choriomeningitis Virus (LCMV), or West Nile Virus (6, 9-11). *Drak2*-deficient mice also develop overtly normal Tmem populations with enhanced function on a per cell basis following MHV infection (10).

In this study, we hypothesized that DRAK2 blockade may serve as a novel approach to alter T cell alloresponses. This notion was prompted by previous studies revealing that blockade of signaling molecules that promote autoimmune susceptibility often are also required for T cell alloreactivity (12, 13). Using two different allogeneic transplant models, we have observed that loss of DRAK2 leads to a significant impairment in allograft rejection. Likely a consequence of premature effector T cell death following allogeneic stimulation, loss of DRAK2 signaling drastically reduced the development of allogeneic Tmem, supporting the contention that long-term allograft maintenance may be achieved through DRAK2 blockade. CTLA4-Ig synergized with DRAK2 blockade, leading to enhanced allograft survival in a stringent skin transplant model. These discoveries underscore the significance of DRAK2 blockade in achieving allograft tolerance, and support an alternative means to promote allograft survival without imposing stringent immunosuppression.

Materials and methods

Mice and Reagents

Drak2-/- mice were backcrossed onto the C57BL/6J ($H2^b$) background for more 10 generations and have been described previously (6). Bcl-xL transgenic mice were generated in the Craig Thompson lab and were crossed with *Drak2*-/- mice to generate *Bcl-xL* × *Drak2*-/- mice (14). *Rag2*-/- × IL2Rγ C-/- mice were purchased from Taconic (Hudson, NY). Animal studies were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Reagents used for flow cytometry included anti-H2d, anti-Interferon-gamma (IFNg, anti-CD8, anti-CD4, anti-KLRG1, and anti-CD127 (Biolegend, San Diego, CA), 7-Aminoactinomycin D (7AAD), and carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA). Intracellular IFNg staining has been described previously (7).

Allogeneic Tumor Transplants and Skin Engraftment

Rag2-/- × IL2Rγ C-/- mice were adoptively transferred by retro-orbital injection with 2.5×10^6 pure naïve T cells or 1×10^5 Tmem. Homeostatic proliferation of T cells was permitted for 7d prior to tumor transplantation. Allogeneic L1210 ($H2^d$) tumors were instilled subcutaneously under the flanks. Allogeneic skin from Balb/c ears was placed onto the flanks of WT or *Drak2*-/- mice following a $1cm^2$ incision. Skin was secured by sterile bandage for 6 days. For adoptive transfer model, *Rag2*-/- × IL2Rγ C-/- mice were transplanted with skin first, followed by adoptive transfer of 2.5×10^6 wildtype or *Drak2*-/- purified T cells 6 days later. For CTLA4-Ig treatment, mice were injected with 500 micrograms on day 0, 2, 4, 6, and 8 following adoptive transfer.

Results

In order to determine if loss of DRAK2 altered T cell alloresponses, mixed lymphocyte reactions (MLRs) were performed. After culturing purified CFSE-labeled *Drak2-/-* T cells ($H2^b$) with allogeneic Balb/c ($H2^d$) splenocytes, *Drak2*-/- T cells had diminished accumulation after 5 days (5d) compared to C57BL/6J (WT) cells (**Fig. 1a**). Since *Drak2* alters TCR sensitivity, we tested various ratios of stimulators to responders in an MLR. Both CD4+ and CD8+ T cells from *Drak2*-/- mice exhibited diminished proliferative capacity at various ratios, further demonstrating that allogeneic activation in the absence of DRAK2 signaling results in defective allo-MLR activity (**Fig. 1b**). Earlier time points were also examined, and *Drak2-/-* T cell accumulation defects manifested as early as 3d post activation, although statistically significant differences were not noted until 4d (**Fig. 1c**). MLR cultures were stained with 7AAD following 5d of stimulation to detect non-viable cells, and the ratio of CD8+ cells that died during cycling was compared to live cycling cells (**Fig. 1d**). *Drak2-/-* CD8+ T cells exhibited enhanced apoptosis following allogeneic stimulation, and this defect could be partially rescued by the addition of anti-CD28 Abs, but only modestly with late costimulatory signals through OX40 (**Fig. 1e**).

Drak2-/- T cell alloresponses were tested *in vivo* using an L1210 allogeneic tumor transplant model (15). Wildtype or *Drak2*-/- T cells were magnetically purified and adoptively transferred into *Rag2*-/- × IL2Rγ_C-/- mice that lack T, B and natural killer cells (16). Following adoptive transfer, T cells underwent homeostatic proliferation for 7d prior to allogeneic tumor challenge. No defects were seen in the reconstitution of sham mice with *Drak2*-/- T cells compared to WT (**Suppl. Fig. S1a, b**). Allogeneic L1210 ($H2^d$) tumor cells were inoculated under the flanks of $H2^b$-expressing WT or *Drak2*-/- T cell recipients, and tumor growth was measured over time. Mice that received 2.5×10^6 *Drak2*-/- T cells failed to reject allogeneic tumors, as seen by increasing tumor growth over the course of 12d (**Fig. 2a**). In contrast, mice bearing wildtype T cells displayed rapid rejection of the tumor allografts, with maximal tumor sizes peaking at roughly 7d post transplantation. Prevention of effector T cell (Teff) apoptosis *in vivo* was accomplished by crossing *Drak2*-/- mice with mice carrying a T cell specific *Bcl-xL* transgene (14). Following allogeneic tumor transplantation, *Bcl-xL* expression in *Drak2-/-* T cells restored their ability to promote allorejection (**Fig. 2b**). Tumor growth in the absence of T cells was more rapid than in *Drak2-/-* T cell hosts, although at no time did tumor growth regress when *Drak2-/-* T cells were transferred (**Fig. S2a**). To further examine *Drak2-/-* T cell responses following allogeneic tumor stimulation, splenocytes from WT or *Drak2-/-* mice that had received *in situ* allogeneic tumors 7d prior were retrieved. Day 7 post transplant was chosen as the peak of the rejection response. Tumor rejection *in situ* occured with similar kinetics in *Drak2-/-* mice, possibly due to help from other immune cell populations (**fFig. S2b**). After 5d of secondary restimulation *ex vivo* with L1210 cells, *Drak2-/-* T cell proliferation was almost completely absent (**Fig. 2c**). I*n vitro* restimulation of T cells following tumor rejection at

14d with L1210 tumor cells revealed drastically reduced production of IFNγ by *Drak2-/-* CD8+ cells demonstrating that very few alloreactive Teff were left following initial activation (**Fig. 2d, Fig. S2c**). Response to tumor antigen was not a factor here, since restimulation *ex vivo* with allogeneic splenocytes yielded similar results (**Fig. S2d**). These data support the hypothesis that *Drak2-/-* effector T cells undergo abortive proliferation due to premature cell death following allogeneic activation *in vivo*.

To address the potential that DRAK2 may contribute to allo-specific memory, WT or *Drak2-/-* mice inoculated *in situ* with L1210 tumor cells, and spleens were retrieved 50d later. Purification of allogeneic Tmem was performed by magnetic depletion of antigen presenting cells (APC) and CD45RB+ naïve T cells, followed by adoptive transfer into *Rag2*-/- × IL2Rγ$_C$-/- hosts. While WT Tmem rapidly rejected the tumors, allo-tumor challenge failed to provoke a strong allogeneic response by *Drak2-/-* Tmem (**Fig. 3a**). To further address this point, naïve or Tmem were mixed with allogeneic tumors in an ELISPOT assay for IFN$_\gamma$ Following 48h, naïve *Drak2-/-* T cells formed similar numbers of ELISPOTs vs. WT, suggesting that the initial alloreactive repertoire was normal in *Drak2-/-* mice (**Fig. 3b**). Alternatively, *Drak2-/-* Tmem populations formed significantly fewer ELISPOTs upon restimulation (**Fig. 3b**). Previous reports have defined short-term effectors and long term Tmem populations based on KLRG1 and CD127 surface expression (17). Upon analysis of these populations in *Drak2-/-* mice previously transplanted with L1210 tumors, CD8+ KLRG1- CD127+ long-term Tmem cell numbers were significantly diminished compared to WT (**Fig. 3c**). Differences in the CD4+ memory compartment were noted, although the difference in number of CD4+ CD127+ Tmem cells was not statistically significant. These data highlight the overall defective allospecific Tmem response seen in the *Drak2-/-* CD8+ population.

In experimental transplantation, skin transplants are considered one of the most stringent tests for allogeneic transplant tolerance in mice (18). Following successful engraftment of ear skin from allogeneic Balb/c (H2^d) mice onto the backs of C57BL/6J or *Drak2*-/- mice, signs of graft rejection were monitored daily. Wildtype recipients rapidly rejected skin grafts (80% necrotic), whereas *Drak2*-/- mice displayed significantly delayed rejection; in some cases, transplant maintenance was equivalent or more prolonged than a previous report using CTLA4-Ig to block costimulation (**Fig. 4a**) (4). Although rejection did eventually occur in this model, the strong stimulatory properties of skin grafts may have promoted the rescue of *Drak2-/-* Teff and Tmem populations. To address the role of strong allostimulation on *Drak2-/-* memory formation, we collected spleens from mice that had rejected skin grafts 50d prior. Following Tmem purification and transfer into *Rag2*-/- × IL2Rγ$_C$-/- hosts, WT Tmem rapidly rejected skin grafts, whereas *Drak2-/-* Tmem transfer led to a significant delay, and in some instances, a complete lack of rejection (**Fig. 4b**). Examination of the phenotype of the Tmem populations in *Drak2-/-* mice revealed significantly fewer long lived KLRG1^{Lo}/CD127^{Hi} memory cells in the CD8+ compartment, and a nearly 3 fold increase in KLRG1^{Hi}/CD127^{Hi} cells for both CD4+ and CD8+ subsets vs. WT mice (**Fig. 4c,d**). We also noted diminished recovery of CD8+ CD44+ CD62L+ central memory cells, while no differences were seen in the CD44+ CD62L- effector memory population (**Fig. S3a,b**). Further, these cells expressed comparable levels of PD1, suggesting that the diminished function of Drak2-/- Tmem was not due to clonal exhaustion (**Fig. S3c**). Previous reports have suggested these KLRG1^{Hi}/CD127^{Hi} cells have an intermediate life span following viral infection, although future investigation will be aimed at determining the functional capacity of these cells during allograft rejection (17).

Since addition of costimulatory anti-CD28 antibodies (Abs) to MLR cultures enhanced DRAK2 allo-responsiveness, we were curious about the potential synergistic effects of blocking both DRAK2 signaling and CD28-mediated costimulation during an alloresponse.

Thus, we treated WT and *Drak2-/-* mice with CTLA4-Ig on days 0, 2, 4, 6, and 8 following allogeneic Balb/c skin transplant, and assessed graft survival over time. Blocking CD28-mediated costimulation with CTLA4-Ig increased graft survival time of WT mice by several days, a rescue similar to that observed in untreated *Drak2-/-* mice (**Fig 5a**). However, the combination of a *Drak2* deficiency along with costimulatory blockade led to significantly longer graft survival times, with a majority of skin grafts still intact after 28d. To specifically address the T cell intrinsic requirements for DRAK2 signaling during skin transplant rejection, *Drak2-/-* T cell alloresponses to Balb/C skin grafts in an adoptive transfer model were investigated. First, *Rag2*-/- IL2Rγ_C-/- mice were transplanted with Balb/c skin grafts, followed by adoptive transfer of purified WT or *Drak2-/-* T cells 6d later. Mice bearing *Drak2-/-* T cells exhibited significantly delayed skin graft rejection compared to those bearing WT T cells (**Fig. 5b**). Again, CTLA4-Ig was administered immediately following adoptive transfer, and continued every other day until 8d post adoptive transfer. Similar to the *in situ* model of skin transplantation shown earlier, *Drak2-/-* T cell recipients treated with CTLA4-Ig maintained skin grafts up to 36d without any signs of rejection (**Fig. 5b**). These findings support the hypothesis that blockade of DRAK2 and CD28 signaling synergistically promotes allograft maintenance.

Discussion

In this report, we demonstrate that DRAK2 signaling is required for productive T cell alloresponsiveness in two separate allogeneic transplant models. These data support the hypothesis that DRAK2 signaling is required for efficient allorejection, and that in the absence of DRAK2 signaling, premature Teff cell death leads to selective deletion of alloreactive clones prior to Tmem formation (**Fig. S4**). Importantly, this survival defect was most pronounced when *Drak2*-/- T cells were activated in the presence of low levels of costimulation. Based on previous work (7), we speculate that the highly inflammatory nature of completely MHC mismatched skin transplants provides an environment favorable to the survival of a fraction of alloreactive Drak2-/- Teff capable of eventual graft rejection. In agreement with this, we observed complete graft acceptance by *Drak2-/-* mice using an allogeneic tumor model, whereas skin transplants were eventually rejected, albeit with delayed kinetics. The allogeneic tumor cell populations used in this study have diminished stimulatory properties when compared to skin grafts since they do not harbor allogeneic professional APCs, which can greatly enhance alloresponses (18). Skin transplants survived significantly longer when DRAK2 signaling was absent from T cells, but the most profound graft survival was observed when DRAK2 and CD28 signaling were simultaneously lost. We did not see appreciable effects when OX40 costimulation was provided *in vitro*, presumably because this source of costimulation is received too late to rescue apoptotic *Drak2-/-* T cells; OX40 expression is low on naïve T cells, and is upregulated several days after antigenic stimulation. We note that these findings are reminiscent of those described in which varying levels of costimulation restored PKC-theta deficient T cell responses to allogeneic hearts (13).

In addition to providing acute tolerance to allogeneic transplants, an essential goal is to prevent chronic allograft rejection without imposing long-term blockade of conventional immune responses. In order to maintain allograft survival over long periods of time (e.g. the remaining lifetime of a transplant recipient), Tmem responsive to alloantigen must be repressed or deleted. In these studies, we demonstrate that initial interaction of *Drak2*-/- T cells with alloantigen leads to deletion of alloreactive T cells, and as a consequence, drastically affects both short- and long-term recall responses. As early as 14d post activation, *Drak2*-/- T cells failed to efficiently respond to allogeneic tumors. Even 50d post transplantation, IFNγ production was significantly diminished in *Drak2-/-* Tmem re-challenged with alloantigen. Previous reports have linked suboptimal T cell activation to

diminished memory pool formation in viral and altered peptide ligand models, and these data further stress the importance of optimal T cell activation conditions to provide long-term Tmem survival (19, 20). W e have previously demonstrated that DRAK2 is not required for the generation of antiviral Tmem, perhaps due to the highly inflammatory nature of an antiviral response (10). In contrast, DRAK2 is vital for alloreactive Tmem generation. In both transplant models tested, *Drak2-/-* T cell populations possessed drastically reduced KLRG1- CD127+ Tmem pools, providing evidence that DRAK2 blockade may not only impact acute rejection, but also help minimize chronic rejection mediated by alloreactive memory cells.

As DRAK2 is a protein kinase, it is plausible that small molecule antagonists may be synthesized for specifically blocking its intracellular activity. Our findings suggest that DRAK2 blockade may have the greatest therapeutic potential when CD28-mediated costimulatory signaling is limited either by availability on the surface of the graft due to the type of transplant or by administration of CTLA4-Ig. Consequently, recipients of weakly immunogenic grafts such as pancreatic islets or stem cells may benefit most from merely blocking DRAK2 alone, whereas vascularized tissue and organ transplants bearing allogeneic APCs may require combination therapies in which both DRAK2 and CD28 signaling are inhibited. Distinct from current immunosuppressive regimens, DRAK2 blockade may thus promote the selective deletion of alloreactive T cell clones and prevent memory formation without negatively impacting antiviral immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

7AAD	7-Amino Actinomycin D
Abs	antibodies
ANOVA	analysis of variance
APC	antigen presenting cell
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTLA4-Ig	cytotoxic T-lymphocyte antigen 4 fusion protein immunoglobulin
d	days
ELISPOT	enzyme-linked immunosorbent spot
IFNg	interferon gamma
LCMV	lymphocytic choriomeningitis virus
MHV	murine hepatitis virus
MLR	mixed lymphocyte reaction
n	number in group

Tmem memory T cell

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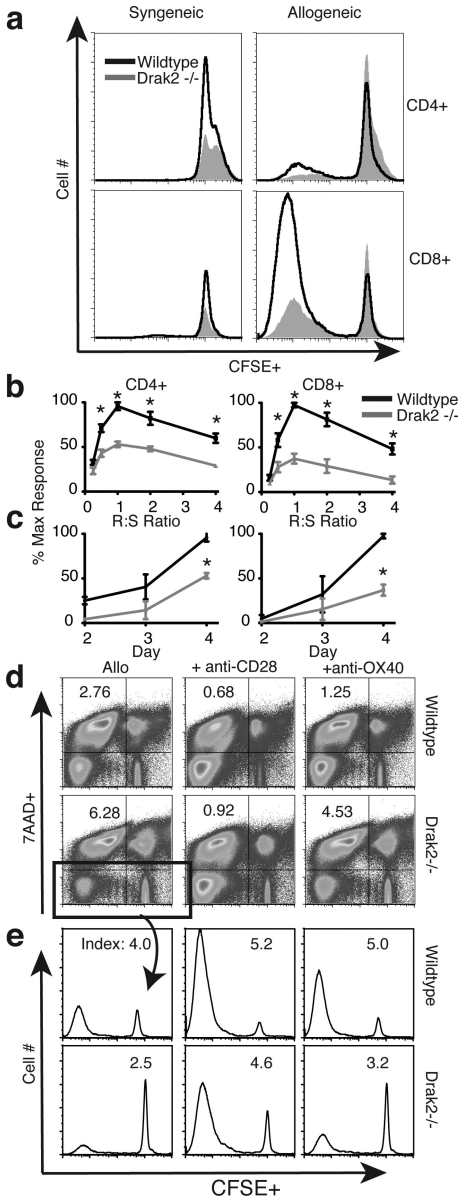

Figure 1.

Drak2-/- T cells exhibit defective alloresponses following a mixed lymphocyte reaction. (a) Wildtype or *Drak2*-/- purified T cells were CFSE labeled and then mixed with allogeneic Balb/c splenocytes for 5d prior to analysis by FACS. Stimulators were excluded by H2d+ staining, CD4+ and CD8+ cells were gated, and CFSE dilution was determined. (b) Purified T cells were mixed at various ratios with Balb/c stimulators. Percent maximum response was determined by dividing the number of CFSE low cells from each condition by the maximum CFSE low cell number for the entire experiment. *P<0.05 by unpaired student's T test, n=5. (c) MLR (1:1) analyzed at various time points. *P<0.05 by unpaired student's T test, n=3. (d) Following 5d MLR, dead cells were stained with 7AAD and analyzed by FACS. The ratio of CD8+ CFSE low, dead to live cells is shown. (e) Additional gating from panel (d) on the 7AAD negative population with proliferation index shown (all plots same scale). Each panel exhibits representative data from a minimum of 9 (a) or 3 (b-e) independent experiments.

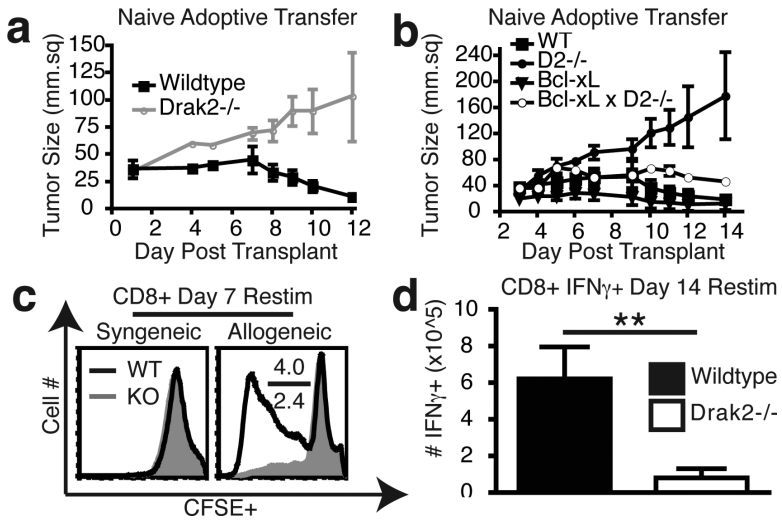

Figure 2.

Drak2-/- T cells cannot reject allogeneic tumors *in vivo* due to enhanced effector cell death. (a) 2.5 × 10^6 purified Wildtype or *Drak2*-/- T cells were transferred into *Rag2*-/- × IL2Rγ C-/- hosts 7 days prior to transplant. 20 × 10^6 allogeneic ($H2^d$) L1210 tumor cells were implanted under the flanks of mice, and rejection assessed by daily tumor growth measurements. Data shown are mean +/- SEM. ***P<0.0001 by ANOVA, n = 9. (b) Purified T cells from the 4 different genotypes were transferred into *Rag2*-/- × IL2Rγ C-/- hosts 7 days prior to transplant. 20 × 10^6 allogeneic L1210 tumor cells were implanted, and rejection was monitored daily; mean +/- SEM shown. ***P<0.001 by ANOVA, n=3. (c) CFSE labeled splenocytes from WT or *Drak2-/-* mice that had received *in situ* allogeneic tumors 7d prior were restimulated *ex vivo* with L1210 tumor cells, stained with CD8 Abs, and proliferation was measured by CFSE dilution. Proliferation index is labeled. (d) Splenocytes recovered from *in situ* tumor transplanted mice at d14 were rechallenged with L1210 tumor cells for 36h prior to intracellular staining for $IFN_g^{\sim}$ **P<0.01 by unpaired student's T test, n = 3 each.

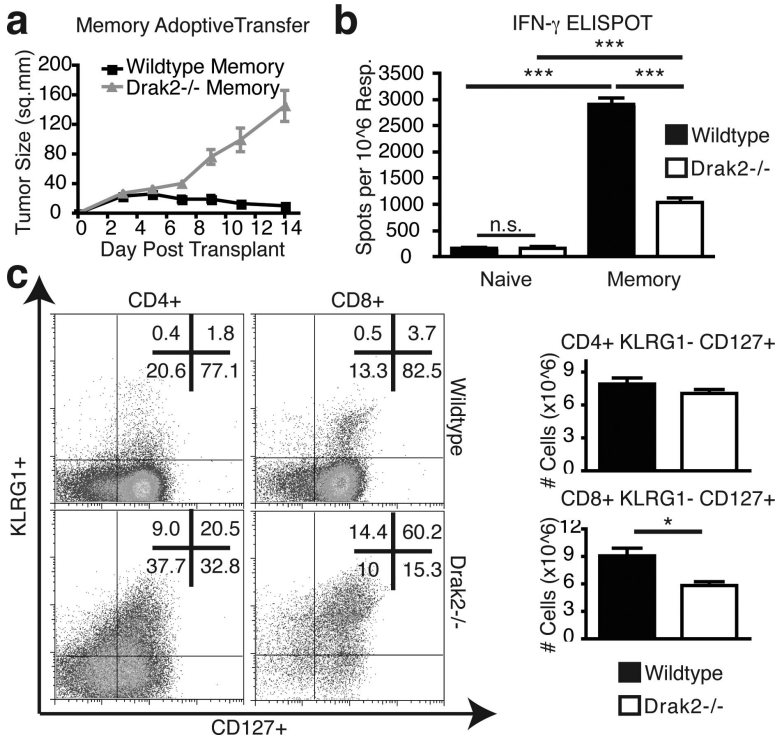

Figure 3.

Drak2-/- memory pool formation is defective following allogeneic tumor transplantation. (a) 2.5×10^6 T cells were purified from naïve mice, or mice previously exposed to allogeneic tumors *in situ* for 50d and then transferred into *Rag2*-/- × IL2Rγ C-/- hosts. Tmem were enriched by CD45RB depletion for the memory condition. Mice were challenged with 20×10^6 L1210 tumor cells for 14d. Error bars represent +/- SEM. ***P<0.0001 by ANOVA, n = 5. Data representative of 2 separate experiments. (b) Purified T cells from naive, or previously challenged (memory) mice were mixed in 96 well ELISPOT plates, and IFNg was captured and detected after 48h culture. ***P<0.001 using unpaired student's T test. (c) Wildtype or *Drak2*-/- mice were inoculated with 20×10^6 allogeneic L1210 cells *in situ* 50d prior to retrieving spleens. Splenocytes were labeled with KLRG1, CD127, CD4, and CD8 Abs prior to FACS. *P<0.02 using unpaired student's T test.

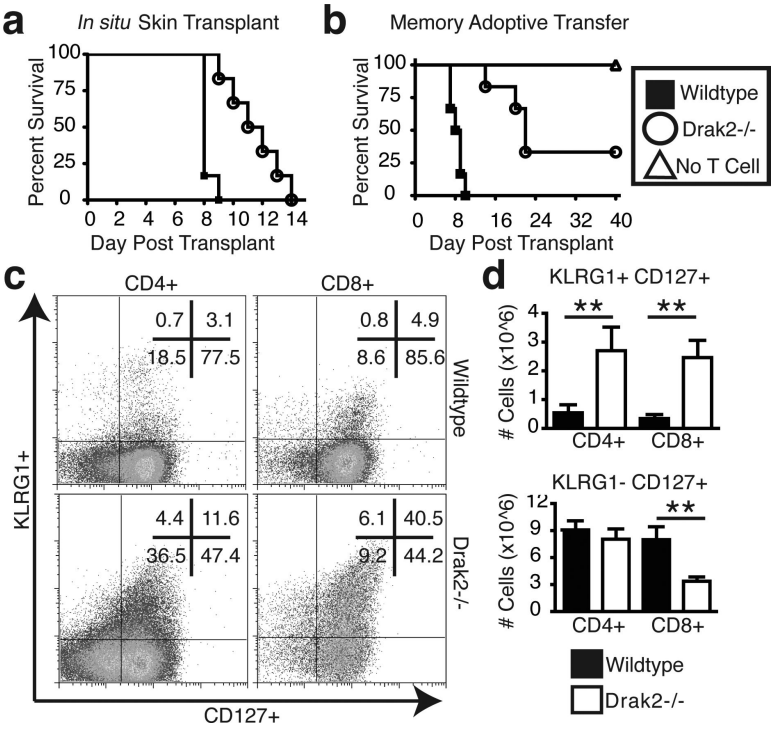

Figure 4.

Drak2 deficiency leads to enhanced survival of allogeneic skin grafts, and decreased allogeneic memory. (a) WT or *Drak2*-/- mice were transplanted *in situ* with ear skin from allogeneic Balb/c mice. Graft survival was monitored daily and considered rejected when 80% necrotic. *P<0.001, n = 5 WT and 6 *Drak2*-/-. (b) Allogeneic Balb/c skin was transplanted onto WT or *Drak2-/-* mice 50d prior. Tmem were enriched by CD45RB depletion from spleens, and transferred into *Rag2*-/- × IL2Rγ C-/- hosts that had received skin grafts 6d prior. *P<0.001, n = 6 each. (c) Wildtype or *Drak2*-/- mice were transplanted with allogeneic skin 50d prior to retrieving spleens. Splenocytes were labeled with KLRG1, CD127, CD4, and CD8 Abs prior to FACS. **P<0.03 and ***P<0.003 using unpaired student's T test.

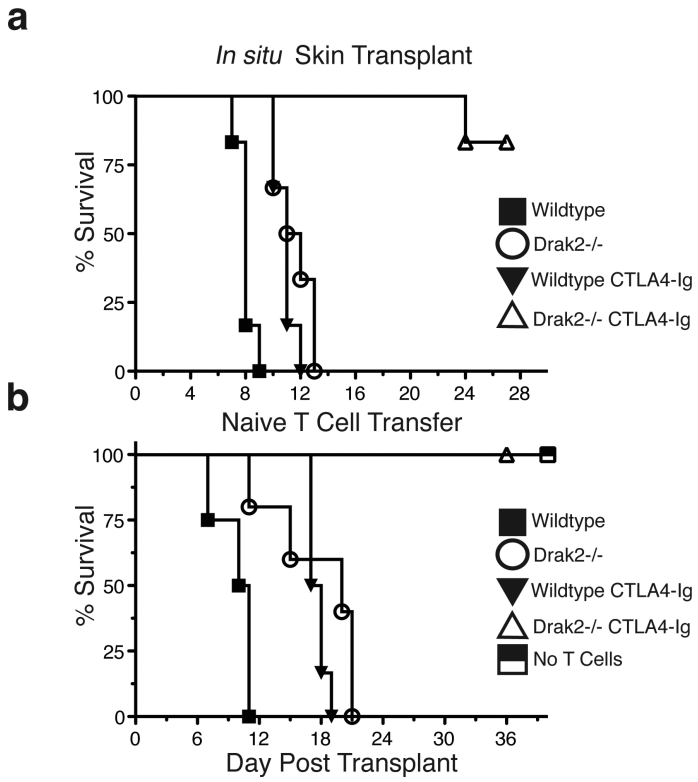

Figure 5.

Costimulation blockade using CTLA4-Ig synergizes with *Drak2* deficiency leading to long term allogeneic skin graft survival. (a) WT or *Drak2*-/- mice were transplanted with ear skin from allogeneic Balb/c mice and treated with CTLA4-Ig starting on day of transplant until d8. ***P<0.001, n=5 WT and *6 Drak2-/-* untreated, n=6 CTLA4-Ig treated. (b) WT or *Drak2*-/- purified T cells were transferred into *Rag2-/-* × IL2Rγ C-/-hosts that had previously received allogeneic skin transplants 6d prior. Mice were treated with CTLA4-Ig starting on day of adoptive transfer until d8. P<0.001, n=4-6 mice per condition.