Dickkopf-3, an immune modulator in peripheral CD8 T-cell tolerance

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In healthy individuals, T cells react against incoming pathogens, but remain tolerant to self-antigens, thereby preventing autoimmune reactions. CD4 regulatory T cells are major contributors in induction and maintenance of peripheral tolerance, but a regulatory role has been also reported for several subsets of CD8 T cells. To determine the molecular basis of peripheral CD8 T-cell tolerance, we exploited a double transgenic mouse model in which CD8 T cells are neonatally tolerized following interaction with a parenchymal self-antigen. These tolerant CD8 T cells have regulatory capacity and can suppress T cells in an antigen-specific manner during adulthood. Dickkopf-3 (Dkk3) was found to be expressed in the tolerant CD8 T cells and to be essential for the observed CD8 T-cell tolerance. In vitro, genetic deletion of Dkk3 or blocking with antibodies restored CD8 T-cell proliferation and IL-2 production in response to the tolerizing self-antigen. Moreover, exogenous DKK3 reduced CD8 T-cell reactivity. In vivo, abrogation of DKK3 function reversed tolerance, leading to eradication of tumors expressing the target antigen and to rejection of autologous skin grafts. Thus, our findings define DKK3 as a immune modulator with a crucial role for CD8 T-cell tolerance.

Tolerant CD8 T Cells Express DKK3. In Des.KKb mice, tolerance to the self-antigen Kb is mediated by a CD8 T-cell population with a regulator of peripheral T-cell tolerance, quantitative RT-PCR analysis of ex vivo isolated tolerant, naive, and activated Des CD8 T cells was performed for gene products involved in tolerance induction (15). Naive Des CD8 T cells were isolated from untreated Des.Rag2−/− mice. Activated Des CD8 T cells were derived from Des.Rag2−/− mice, which had rejected a Kb-positive tumor graft, whereas tolerant Des CD8 T cells were derived from Des.KKb.Rag2−/− mice, which had failed to reject a Kb-positive tumor. (Fig. S1). All mice were thymectomized at 12 d of age to enrich for neonatally derived Des CD8 T cells (13). As DKK3 is highly expressed in tissues classically termed “immune privileged” (20, 21), Dkk3 was included in the RT-PCR analysis. A 10- and 15-fold up-regulation of Dkk3 expression in tolerant Des CD8 T cells was found, compared with naive and activated T cells, respectively (Fig. 1A). Intracellular staining with a newly established DKK3-specific monoclonal antibody (Fig. S2) indicated a similar pattern at the protein level (Fig. 1B). No DKK3 expression was seen in naive C57BL/6 T cells (Fig. S3). Thus, tolerant Des CD8 T cells express DKK3.

Here, we investigated the molecular basis of CD8 T-cell tolerance observed in Des.KKb mice and found Dickkopf-3 (Dkk3) to be expressed in tolerant Des CD8 T cells. DKK3 belongs to the Dickkopf family of secreted proteins (Dkk1–4) that modulate Wnt signaling (16, 17). DKK3 has been reported to suppress tumor cell proliferation in vitro (18, 19). However, the physiological function of this evolutionarily conserved molecule is unknown, as no gross morphologic and phenotypic alterations were found in Dkk3−/− mice (20).

The present study demonstrates that soluble DKK3 can efficiently inhibit T-cell proliferation and IL-2 production in response to in vitro antigen stimulation. Interestingly, abrogation of DKK3 function resulted in loss of CD8 T-cell tolerance in vivo. Furthermore, DKK3 produced by CD8 T cells was sufficient to mediate peripheral CD8 T-cell tolerance in a DKK3-deficient host. Taken together, our data demonstrate a crucial role for DKK3 in peripheral CD8 T-cell tolerance.
Loss of Antigen-Specific T-Cell Hyporesponsiveness in the Absence of DKK3. To assess whether DKK3 is functionally linked to peripheral CD8 T-cell tolerance, we crossed Dkk3−/− mice to Des and Des.KKb transgenic mice. Genetic deletion of DKK3 did not result in any significant alterations in the development of Des CD8 T cells in the thymus (Fig. S4 A–C), nor did it affect the total number of lymphocytes in the spleen of Des.KKb mice (Fig. S4D). Des CD8 T cells were isolated from untreated Des, Des.KKb, Dkk3−/−Des, and Dkk3−/−Des.KKb mice, and their proliferation and IL-2 secretion was assessed following in vitro antigen stimulation.

CD8 T cells from Des mice proliferated after stimulation with irradiated Kb-positive Rag2−/− splenocytes, whereas proliferation of CD8 T cells from Des.KKb mice was reduced (Fig. 1C and Fig. S5A), suggesting that Des CD8 T cells from Des.KKb mice are hyporesponsive. In contrast, such a hyporesponsiveness was not observed in the proliferation of CD8 T cells from Dkk3−/−Des.KKb mice (Fig. 1D and Fig. S5B). Hyporesponsiveness of CD8 T cells from tolerant Des.KKb mice was associated with decreased cell survival 2 d after in vitro antigen stimulation (Fig. 1E and F). Thus, CD8 T cells from Des.KKb mice exhibit hyporesponsiveness and undergo apoptosis following antigen stimulation, and these effects depend on DKK3.

As IL-2 is a major T-cell growth factor contributing to proliferation and differentiation of T cells, we analyzed its production by the different T-cell populations. CD8 T cells from Dkk3−/−Des.KKb mice produced more IL-2 in response to Kb-positive Rag2−/− splenocytes, in terms of frequency (Fig. 1G, Upper) and total amount (Fig. 1G, Lower), compared with Des CD8 T cells from Des.KKb mice. Furthermore, CD8 T cells from Dkk3−/−Des.KKb mice showed increased expression of the activation markers CD25 and CD69 compared with CD8 T cells from Des.KKb mice (Fig. S6). Taken together, DKK3 contributes to the hyporesponsiveness of CD8 T cells from tolerant Des.KKb mice.

Loss of Tolerance in the Absence of DKK3 Is Not Associated with a General T-Cell Hyperreactivity. We next investigated whether loss of DKK3 function entails a general T-cell hyperreactivity leading to abrogation of peripheral tolerance. When in vivo cytotoxicity was measured in single transgenic Des and Dkk3−/−Des mice after stimulation with activated C57BL/6 dendritic cells, Kb-positive, carboxyfluorescein succinimidyl ester (CFSE) labeled target cells were eliminated equally well in both mouse groups (Fig. 2A). To assess whether such effects could also be observed in the general repertoire, ovalbumin (OVA)-specific T-cell responses were assessed in wild-type and Dkk3−/−C57BL/6 mice. The effector function of OVA-specific CD8 T cells was assessed after immunization either with OVA-loaded dendritic cells (Fig. 2B) or with OVA-expressing adenovirus (Fig. 2C). Wild-type and Dkk3−/− mice developed comparable reactivity in both cases.
Moreover, lack of DKK3 did not influence the expansion of OVA-specific CD8 T cells in response to OVA-expressing adenovirus (Fig. 2C). Thus, the in vivo reactivity of nontolerant CD8 T cells in Dkk3−/− mice is comparable with the in vivo reactivity of wild-type CD8 T cells.

Similarly, when Des CD8 T cells from Des and Dkk3−/− mice were stimulated in vitro with irradiated Rag2−/− splenocytes (Fig. 2D), no difference in their proliferation was observed. Moreover, although splenic T cells from Dkk3−/− mice displayed increased proliferation compared with their wild-type controls following suboptimal stimulation with low doses of CD3- and CD28-specific antibodies, this effect was not observed following stimulation with higher antibody concentrations. (Fig. 2E). Thus, Dkk3−/− CD8 T cells are hypersensitive in vitro to weak TCR stimulation in vitro, but are not hyperreactive following antigen stimulation in vitro and in vivo.

**Exogenous DKK3 Inhibits CD8 T-Cell Responses in Vitro.** As DKK3 is a secretory molecule, we next investigated the effect of exogenous DKK3 on in vitro CD8 T-cell responses to their target antigen. Purified naive Des CD8 T cells were stimulated with irradiated Kb-positive Rag2−/− splenocytes in the presence of HEK 293T cells that expressed DKK3 or untransfected HEK 293T cells. Proliferation of Des CD8 T cells was reduced in the presence of DKK3 (Fig. 3A). To assess whether such effects could also be observed in a primary cell line, we used primary cultures of astrocytes, which produce high levels of Dkk3 when cultured in vitro (Fig. 3B). CD8 T cells, stained with CFSE and activated against the Kb antigen, showed an impaired proliferative capacity in the presence of DKK3-secreting astrocytes (Fig. 3B). This inhibitory effect was reversed by addition of a blocking DKK3-specific antibody (Fig. 3B). Furthermore, DKK3-containing supernatant of HEK 293T:Dkk3 cells limited the differentiation of Des CD8 T cells into CD25-expressing, IL-2-secreting effector cells after Kb stimulation in comparison with supernatant of untransfected HEK 293T cells (Fig. 3C). These data indicate that secreted DKK3 can directly act on CD8 T cells and down-modulate their proliferation and IL-2 production.

**Dkk3 Is Indispensable for CD8 T-Cell Tolerance in Des.Kb Mice.** DKK3 is expressed by tolerant CD8 T cells and can suppress T-cell reactivity in vitro. Therefore, we investigated whether DKK3 is essential for the observed CD8 T-cell tolerance in vivo. As readout systems, we used the capacity of Des CD8 T cells to
Cells, unlike Des.KKb mice, in which the peripheral repertoire is strongly skewed toward the Kb specificity. These findings demonstrate that DKK3 is essential for the maintenance of the observed peripheral T-cell tolerance in adult mice, and its blockade results in reversal of the tolerant state.

**Discussion**

The present study demonstrates a role for DKK3 in downregulating CD8 T-cell reactivity. Notably, taking advantage of a well-characterized transgenic mouse model of antigen-specific CD8 T-cell tolerance, we show that (i) DKK3 expression is high in tolerant CD8 T cells; (ii) DKK3 dampens the responsiveness of tolerant CD8 T cells in vitro; (iii) soluble DKK3 inhibits CD8 T-cell responses to their target antigen; (iv) DKK3 is essential for the maintenance of the observed CD8 T-cell tolerance; and (v) DKK3-producing tolerant CD8 T cells can regulate CD8 T cells with the same antigen specificity in a DKK3-deficient environment and, thus, transfer tolerance to their cognate antigen.

Until now, DKK3 expression in T cells has been controversial in the literature. In some reports, DKK3 expression in lymphoid tissues and in leukocytes was below detection level (21, 24). Similarly, we could not find significant amounts of DKK3 protein in naive or activated T cells. In contrast, very sensitive differential gene expression analyses traced DKK3 mRNA in long-term

**Fig. 4.** Dkk3 is indispensable for CD8 T-cell tolerance in vivo. (A) Des (n = 23), Des.KKb (n = 19), and Dkk3−/−.Des.KKb (n = 21) mice were inoculated s.c. with 2 × 10⁵ DKK3-negative P815.Kb.B7 tumor cells. Tumor sizes (mean ± SEM) pooled from two independent experiments (P < 0.0001, Mann–Whitney U test) are shown. (B) The percentage of Des.KKb and Dkk3−/−.Des.KKb tumor-bearing mice at day 21 is shown (P < 0.05, Fisher’s exact test). (C) Des, Des.KKb, and Dkk3−/−.Des.KKb mice were immunized with activated H-2b dendritic cells, and the percentage of lysis in vivo of CFSEhigh Kb-positive targets was measured on day 7 after immunization. Cumulative data from three independent experiments are shown (cytotoxicity: mean ± SEM), P < 0.001, t test of arcsin-transformed values; n = 15/group). (D) Des.KKb mice were transplanted with autologous skin and received 1 mg of either anti-Dkk3 or isotype control antibody i.p. at day 1 followed by 0.5 mg antibody every third day (n = 12 mice per group; P = 0.004, log-rank test).
memory CD8 T cells in the mouse (25). In addition, comparison of gene expression in human leukocytes identified DKK3 as one of the five genes uniquely expressed in CD8 T cells (26). In light of our finding, we suggest that DKK3 expression may be restricted only to subsets of tolerant CD8 T cells.

The relative selectivity of DKK3 expression in tolerant Des CD8 T cells suggested that the molecule may be essential for the maintenance of the observed antigen-specific peripheral T-cell tolerance. A general state of hyperresponsiveness in DKK3-deficient T cells was not found in the transgenic Des and the polyclonal C57BL/6 T-cell repertoire as tested by in vitro K\(^+\)-specific proliferation and in vivo cytotoxicity assays. Because the same conditions were used to evaluate T-cell reactivity in vitro and tolerance in vivo in the respective double-transgenic mice, it is unlikely that hyperresponsiveness of DKK3-deficient T cells can account for the loss of tolerance in Dkk3\(^+-\)Des.KK\(^b\) mice. This view is supported by the reversal of tolerance in Des.KK\(^b\) mice treated with the DKK3-specific antibody. Therefore, the nontolerant phenotype of Dkk3\(^+-\)Des.KK\(^b\) mice cannot be attributed to a deficit during tolerance induction or to an intrinsic defect of Dkk3\(^+-\)Des CD8 T cells. On the contrary, soluble DKK3 is indeed essential for the maintenance of the observed tolerance. Nevertheless, Dkk3\(^+-\) mice on the C57BL/6 background do not spontaneously develop autoimmune diseases (20).

Whether the occurrence of auto-reactivity in Dkk3\(^+-\) mice may depend on the genetic background as found for other gene products involved in tolerance (27), and whether induced autoimmune diseases in Dkk3\(^+-\) mice show enhanced pathology in comparison with wild-type mice, warrant further investigation.

Our previous work showed that in vivo deletion of Des CD8 T cells in 12-d-old Des.KK\(^b\) mice abrogated tolerance in the respective adult mice. We concluded that maintenance of tolerance is based on the regulatory capacity of the neonatally induced tolerant Des CD8 population (13). Therefore, we asked whether DKK3 produced by these T cells is capable per se of mediating tolerance. To mimic the situation in Des.KK\(^b\) mice, we transferred purified Des CD8 T cells from day 12 thymectomized Des.KK\(^b\).Rag2\(^/-\) mice or from day 12 TC8 T cells were transferred. One day later, mice were inoculated s.c. with 2 \(\times 10^5\) P815. K\(^b\).B7 tumor cells. (B) Kinetics of tumor growth in recipients of Des.KK\(^b\) (Left) and TC8 (Right) T cells. One (\(n = 7\)) of two independent experiments is shown. (C) Cumulative data of both experiments showing percentage of tumor-bearing mice (Left) (\(P < 0.05\), Fisher’s exact test) and tumor size (Right) of TC8 and Des.KK\(^b\) recipients (mean \(\pm\) SEM, \(P < 0.01; n = 13\)). (D) Dkk3\(^+-\). Des.KK\(^b\) mice were treated and obtained CD8 T cells from day 12 thymectomized Des.KK\(^b\).Rag2\(^/-\) mice as in A. In addition, these mice received 1 mg of either anti-Dkk3 or isotype control antibody i.p. at day 12 followed by 0.5 mg antibody every third day. Cumulative data of two independent experiments show percentage of tumor-bearing mice (Left) (\(P < 0.05\), Fisher’s exact test) and tumor size (Right) (mean \(\pm\) SEM, \(P < 0.01; n = 12\)).

**Fig. 5.** DKK3-producing tolerant Des CD8 T cells are sufficient to mediate tolerance. (A) Dkk3\(^+-\). Des.KK\(^b\) mice were treated on days -5 and -3 with 0.5 mg anti-CD4 (GK1.5) and 0.5 mg anti-CD8 (33.6.7) depleting antibodies. At the time of T-cell transfer (day 0) less than 1\% of the original CD4 and CD8 T cells was left in the blood of the treated mice. Purified CD8 T cells (10\(^6\)) either from day 12 thymectomized Des.KK\(^b\).Rag2\(^/-\) or from day 12 TC8 T cells were transferred. One day later, mice were inoculated s.c. with 2 \(\times 10^5\) P815. K\(^b\).B7 tumor cells. (B) Kinetics of tumor growth in recipients of Des.KK\(^b\) (Left) and TC8 (Right) T cells. One (\(n = 7\)) of two independent experiments is shown. (C) Cumulative data of both experiments showing percentage of tumor-bearing mice (Left) (\(P < 0.05\), Fisher’s exact test) and tumor size (Right) of TC8 and Des.KK\(^b\) recipients (mean \(\pm\) SEM, \(P < 0.01; n = 13\)). (D) Dkk3\(^+-\). Des.KK\(^b\) mice were treated and obtained CD8 T cells from day 12 thymectomized Des.KK\(^b\).Rag2\(^/-\) mice as in A. In addition, these mice received 1 mg of either anti-Dkk3 or isotype control antibody i.p. at day 12 followed by 0.5 mg antibody every third day. Cumulative data of two independent experiments show percentage of tumor-bearing mice (Left) (\(P < 0.05\), Fisher’s exact test) and tumor size (Right) (mean \(\pm\) SEM, \(P < 0.01; n = 12\)).

Materials and Methods

**Mice.** Des, Des.KK\(^b\), and Dkk3\(^+-\) mice have been described (13, 20). Des and Des.KK\(^b\) were crossed with Dkk3\(^+-\)/C57BL/6 mice. The resulting mice were backcrossed five times to the C57BL/6 background and then intercrossed to homozygosity for the respective transgene and the Dkk3 mutation. Finally, these mice were crossed with Dkk3\(^+-\)/H-2\(d\) mice (backcrosses to DBA/2). Therefore, the resulting H-2\(d\) mice are syngeneic for cells from CBA.KK\(^b\) mice and for the P815.K\(^b\).B7 tumor except for the K\(^d\) antigen. C57BL/6 mice and CBA/K mice were from The Jackson Laboratory. Mice were kept under specific pathogen-free conditions at the German Cancer Research Center animal facility. Experimental procedures were approved by the Regierungsrädsium (Karlsruhe, Germany). Thymectomy and skin transplantation have been previously described in detail (13, 15).

**Generation of Monoclonal Antibody.** HEK 293T-DKK3-igG2b cell supernatant was purified by protein A-Sepharose affinity chromatography. Dkk3\(^+-\) mice were immunized with fusion protein. Splenocytes were fused with cell line x63-Ag8.653. Hybridomas were screened by ELISA for reactivity with a
DKK3-GST fusion protein. Hybridoma Dkk3-4.22 (lgG1 κ-isotype) was cloned by limiting dilution.

Flow Cytometry. Fluorochrome-labeled mAb’s were purchased from BD Biosciences. For intracellular detection, a staining kit was used according to the manufacturer’s instructions (BD Biosciences). Flow cytometric analyses were done on a FACSCanto II.

Real-Time RT-PCR. RNA was isolated using the RNeasy kit (Qiagen) followed by cDNA synthesis with SuperScript II Reverse Transcriptase (Invitrogen). Gene expression levels were determined using real-time PCR TaqMan technology (Applied Biosystems).

Western Blotting. Total protein from 2 × 10^6 cells was separated by SDS–PAGE and transferred to PVDF membranes (Millipore). The membranes were probed with biotinylated anti-DKK3-4.22 antibody and HRP-conjugated streptavidin (Dianova). Detection was performed with SuperSignal West Dura Extended Duration Substrates (Pierce) in a Lumi-Imager (Boehringer).

T-Cell Isolation. Single-cell suspensions from lymphatic organs were separated by negative selection using specific microbeads (Miltenyi Biotec and Invitrogen). To increase purities, magnetic activated cell sorting (MACS)-isolated cells were stained with fluorochrome-labeled antibodies and sorted on a FACSDiva (BD Biosciences).

Western Blotting. Total protein from 2 × 10^6 cells was separated by SDS–PAGE and transferred to PVDF membranes (Millipore). The membranes were probed with biotinylated anti-DKK3-4.22 antibody and HRP-conjugated streptavidin (Dianova). Detection was performed with SuperSignal West Dura Extended Duration Substrates (Pierce) in a Lumi-Imager (Boehringer).

T-Cell Proliferation and IL-2 Assays. Lymphocytes (10^6) were cultured in 96-well plates coated with anti-CD3 and anti-CD28 antibody as indicated. Alternatively, they were stimulated with indicated numbers of irradiated Rag2−/− splenocytes. Where indicated, cells were previously stained with CFSE (0.5 μM) and analyzed at day 3. Supernatants were collected 24 h after stimulation, and the IL-2 concentration was determined by ELISA (Biolinx).

Statistical Analyses. Statistical analyses were performed using by Mann–Whitney U test and log-rank test or as indicated. In all other cases, the two-tailed Student’s t test was used. Error bars denote mean ± SEM. P < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

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Fig. S1. Transgenic experimental model for the isolation of an enriched tolerant CD8 T-cell population. Des and Des.KK<sup>b</sup> mice were deficient for the RAG2 recombinase to exclude other TCR specificities. Furthermore, all mice were thymectomized at 12 d of age to allow exclusion of recent thymic emigrants from the peripheral repertoire and, in the case of Des.KK<sup>b</sup>, to highly enrich for T cells that had been neonatally tolerized. Naive T cells were derived from Des.<sup>Rag2<sup>−/−</sup> mice. Activated T cells were obtained from Des.<sup>Rag2<sup>−/−</sup> mice, which had been grafted at day 70 with P815.K<sup>b</sup>B7.1 tumor cells and had rejected them by day 90. Tolerant T cells were isolated from tolerant Des.<sup>KK<sup>b</sup>Rag2<sup>−/−</sup> mice, which had failed to reject P815.K<sup>b</sup>B7.1 tumor cells by day 90. Spleen and lymph node Des CD8 T cells were isolated on day 90 from all three mouse groups and purified by magnetic activated cell sorting (MACS) magnetic bead enrichment and FACS sorting. Upon mRNA isolation, RT-PCR was performed.

Fig. S2. Characterization of a monoclonal antibody against DKK3. (A) Detection of DKK3 in the cell lysate (CL) and the supernatant (SN) of DKK3-transfected HEK 293T cells by Western blotting with the anti-DKK3 4.22 antibody. Nontransfected HEK 293T cell lysate (CL) was used as a control. (B) Detection of intracellular DKK3 in DKK3-transfected HEK 293T cells by flow cytometry with anti-DKK3 4.22 antibody.
Fig. S3. Tolerant Des CD8 T cells but not naive peripheral CD4 and CD8 T cells express DKK3. (A) Tolerant Des CD8 T cells were isolated from spleen and lymph nodes of adult Des.KKb.Rag2−/− mice, which had been thymectomized at day 12 and either had accepted a Kb-positive tumor graft or were untreated; activated Des CD8 T cells were obtained from adult Des.Rag2−/− mice, which had been thymectomized at day 12 and had rejected a Kb-positive tumor graft. Des CD8 T cells were stained intracellularly with anti-DKK3 4.22 antibody and analyzed by flow cytometry. Mean fluorescence intensity for DKK3 staining is shown. DKK3 expression in tolerant Des CD8 T cells was independent of tumor treatment. Data are pooled from three independent experiments (**P < 0.01, ***P < 0.001; n = 5–6). (B) Spleen and lymph node CD4 and CD8 T cells from C57BL/6 and Dkk3−/− mice were stained intracellularly with anti-DKK3 4.22 antibody and analyzed by flow cytometry. HEK 293T cells that were transfected with DKK3 and EGFP were used as a positive control for DKK3 expression. Representative data from one of three experiments.
Fig. S4. Genetic deletion of DKK3 does not affect thymic development in Des.KKb mice. (A) The percentages of double-negative (DN), double-positive (DP), and single-positive CD4 (SP4) and CD8 (SP8) thymocytes were comparable in Des.KKb and Dkk3−/−Des.KKb mice. (B) The early phases of T-cell development leading to TCRβ selection were unaltered in the absence of DKK3, as assessed by CD44/CD25 staining of DN thymocytes. The percentages of DN1 (CD44+CD25−), DN2 (CD44+CD25+), DN3 (CD44−CD25+) and DN4 (CD44−CD25−) thymocytes were comparable in both types of mice. (C) The expression of CD3 and CD69 on DP thymocytes was unchanged in Des.KKb mice in the presence or absence of DKK3. (D) Genetic deletion of Dkk3 does not alter the number of total spleen cells in Des.KKb mice (P = 0.07; n = 3). Representative results of three independent experiments are shown.
Fig. S5. DKK3 expression is essential for the hyporesponsiveness of tolerant Des CD8 T cells from Des.KKb mice. (A) Carboxyfluorescein succinimidyl ester (CFSE) labeled Des CD8 T cells from Des and Des.KKb mice were stimulated with $10^5$ irradiated splenocytes from Rag2$^{-/-}$ mice, and their proliferation was assessed at day 3 by the dilution of CFSE. Analysis of the proliferation index was performed, and mean values (mean ± SEM) are shown (*$P < 0.05$; $n = 2$). The results are representative of three independent experiments. (B) CFSE-labeled Des CD8 T cells from Dkk3$^{+/+}$ and Dkk3$^{-/-}$ Des.KKb mice were stimulated with 105 irradiated splenocytes from Rag2$^{-/-}$ mice, and their proliferation was assessed at day 3 by the dilution of CFSE. Analysis of the proliferation index was performed, and mean values (mean ± SEM) are shown (**$P < 0.01$; $n = 3$). The results are representative of three independent experiments.

Fig. S6. Up-regulation of the activation marker CD69 and of CD25 upon stimulation of Des CD8 T cells from Dkk3$^{-/-}$. Des.KKb mice. (A) Upon stimulation with irradiated splenocytes from Rag2$^{-/-}$ mice, Des CD8 T cells from Dkk3$^{-/-}$ Des.KKb mice exhibited increased surface levels of CD25 and CD69 compared with Des CD8 T cells from Des.KKb mice. The fold increase of CD25 and CD69 in the surface of Dkk3$^{-/-}$ Des.TCR CD8 T cells compared with Dkk3$^{+/+}$ controls are shown. (B) Expression of CD25 (Upper) and CD69 (Lower) at day 1 after stimulation with irradiated splenocytes from Rag2$^{-/-}$ mice is shown on gated Des CD8 T cells from Des.KKb mice on the Dkk3$^{-/-}$ or Dkk3$^{+/+}$ genetic background. Results are representative of three independent experiments.

Fig. S7. Astrocytes produce high levels of DKK3 in vitro. (A) Lysates from primary astrocyte culture were tested by RT-PCR for dkk3 mRNA. The mean of three independent tests (mean ± SEM) is shown. Naive CD8 T-cell mRNA was used as a negative control. (B) The levels of DKK3 in the supernatant of primary astrocyte cultures from C57BL/6 and Dkk3$^{-/-}$ mice assessed by Western blotting, using the DKK3-specific 4.22 mAb.
Fig. S8. Blockage of DKK3 does not prevent autologous skin graft acceptance in CBA/J mice. CBA/J mice were transplanted with autologous skin and received 1 mg of either anti-DKK3 or isotype control antibody i.p. at day 1 followed by 0.5 mg antibody every third day (n = 12 mice per group; n.s.: not significant, log-rank test).