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.

immune tolerance | parenchymal cells | autoimmunity

A key feature of the immune system is its capacity to eliminate foreign pathogens and tumor cells without harming healthy tissues. A number of finely regulated mechanisms protect the organism from immune-mediated tissue damage and autoimmune diseases. Central tolerance ensures the elimination of high-affinity self-reactive T cells during lymphocyte development by negative selection (1, 2), T-cell receptor (TCR) editing, and anergy induction (3). Harmful reactions against self-tissues are controlled in the periphery by T-cell intrinsic processes including peripheral anergy and deletion (4, 5) and T-cell extrinsic mechanisms such as immunosuppressive cytokines (6) and regulatory T cells. FoxP3$^+$ CD4 T cells are the most prominent regulatory T cells preventing autoimmune reactions (7, 8). Most recently, several lines of evidence have highlighted the essential contribution of CD8 regulatory T cells to peripheral tolerance as well (9–11).

We have previously shown that CD8 T cells with a transgenic K^b-specific TCR (Des) (12) can be tolerized in the periphery and that T-cell tolerization occurs in the neonate following encounter of the K^b antigen exclusively expressed on keratinocytes (KK^b mice) (13). Whereas in adult animals naive T lymphocytes have restricted access to nonlymphoid tissues, neonatal T cells traffic into extralymphoid tissues (14). Deletion of neonatal CD8 T cells in 12-d-old Des.KK^b mice abrogated tolerance, as they rejected a K^b-positive graft, whereas thymectomy at 2 wk of age did not reverse the tolerant state (13). Tolerant Des CD8 T cells were shown to have regulatory capacity, as they down-modulated the reactivity of adoptively transferred naive T cells to the cognate K^b antigen (15).

Here, we investigated the molecular basis of CD8 T-cell tolerance observed in Des.KK^b 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.

Results

Tolerant CD8 T Cells Express DKK3. In Des.KK^b mice, tolerance to the self-antigen K^b is mediated by a CD8 T-cell population with regulatory capacity (15). In identifying molecular mediators of this dominant form 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 obtained from Des.*Rag2*$^{-/-}$ mice, which had rejected a K^b-positive tumor graft, whereas tolerant Des CD8 T cells were derived from Des.KK^b.*Rag2*$^{-/-}$ mice, which had failed to reject a K^b-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. 1*A*). Intracellular staining with a newly established DKK3-specific monoclonal antibody (Fig. S2) indicated a similar pattern at the protein level (Fig. 1*B*). No DKK3 expression was seen in naive C57BL/6 T cells (Fig. S3). Thus, tolerant Des CD8 T cells express DKK3.

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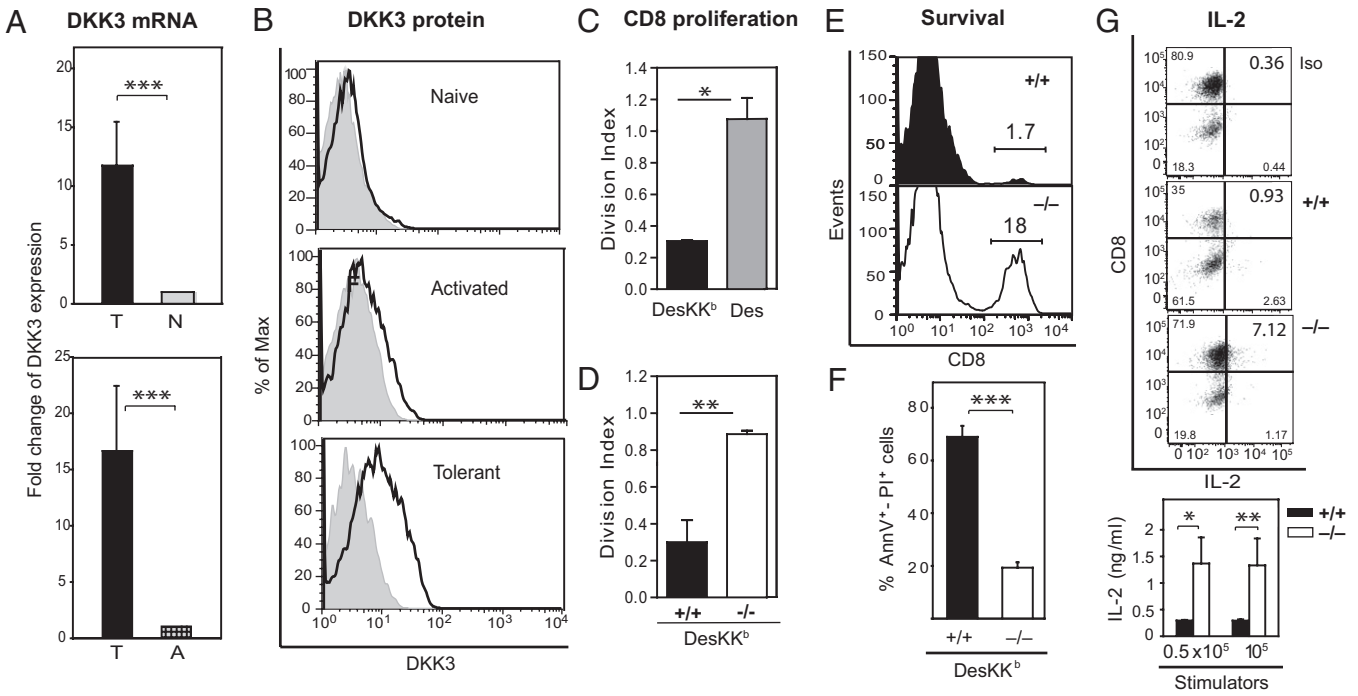

Fig. 1. DKK3 is expressed in tolerant CD8 T cells and is required for their hyporesponsiveness in vitro. (*A*) The levels of *Dkk3* mRNA were quantified by RT-PCR in Des T cells under different in vivo functional states. Tolerant, naive, and activated Des T cells were obtained as depicted in Fig. S1. Naive CD8 T cells were derived from Des.*Rag2*$^{-/-}$ mice; tolerant CD8 T cells were isolated from Des.KK^b. *Rag2*$^{-/-}$ mice, which had accepted a K^b-positive tumor graft; and activated CD8 T cells were obtained from Des.*Rag2*$^{-/-}$ mice, which had rejected a K^b-positive tumor graft. In naive and activated CD8 T cells, DKK3 expression was below detection level. The relative DKK3 expression in tolerant versus naive (*Upper* graph) or activated (*Lower* graph) Des CD8 T cells is shown. Data are representative of three independent experiments ($P < 0.001$; $n = 8$). (*B*) Protein expression was assessed by intracellular staining and flow cytometry [isotype control (gray); anti–DKK3-4.22 antibody (black)]. Data are representative of two independent experiments. Cumulative data are depicted in Fig. S3*A*. (*C*) Proliferation of CFSE-labeled CD8 T cells derived from Des and Des.KK^b mice was assessed 3 d after stimulation with 10^5 irradiated splenocytes from *Rag2*$^{-/-}$ mice. Representative data from three independent experiments are shown. (*D*) Proliferation of CFSE-labeled CD8 T cells from *Dkk3*$^{-/-}$ and *Dkk3*$^{+/+}$.Des.KK^b mice was assessed 3 d following in vitro stimulation with 10^5 irradiated splenocytes from *Rag2*$^{-/-}$ mice. Representative data from three independent experiments are shown. (*E* and *F*) Survival of Des CD8 T cells from *Dkk3*$^{-/-}$ and *Dkk3*$^{+/+}$ Des.KK^b mice was assessed 2 d after in vitro stimulation with 10^5 irradiated splenocytes from *Rag2*$^{-/-}$ mice. Representative percentages of living Des CD8 T cells (*E*: forward/sideward scatter lymphocyte gate) and the mean percentage of apoptotic Des CD8 T cells (*F*) from three independent experiments are shown ($P < 0.001$; $n = 3$). (*G*) CD8 T cells were isolated from *Dkk3*$^{-/-}$ and *Dkk3*$^{+/+}$ Des.KK^b mice and stimulated with irradiated *Rag2*$^{-/-}$ splenocytes. IL-2 was measured by intracellular staining and flow cytometry (*Upper* graph: 10^5 stimulators) and by ELISA in the supernatant (*Lower* graph: $*P < 0.05$, $**P < 0.01$; $n = 3$ mice per group) 24 h after stimulation.

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.KK^b 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.KK^b mice (Fig. S4*D*). Des CD8 T cells were isolated from untreated Des, Des.KK^b, *Dkk3*$^{-/-}$.Des, and *Dkk3*$^{-/-}$.DesKK^b 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 K^b-positive *Rag2*$^{-/-}$ splenocytes, whereas proliferation of CD8 T cells from Des.KK^b mice was reduced (Fig. 1*C* and Fig. S5*A*), suggesting that Des CD8 T cells from Des.KK^b mice are hyporesponsive. In contrast, such a hyporesponsiveness was not observed in the proliferation of CD8 T cells from *Dkk3*$^{-/-}$.Des.KK^b mice (Fig. 1*D* and Fig. S5*B*). Hyporesponsiveness of CD8 T cells from tolerant Des.KK^b mice was associated with decreased cell survival 2 d after in vitro antigen stimulation (Fig. 1 *E* and *F*). Thus, CD8 T cells from Des.KK^b 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.KK^b mice produced more IL-2 in response to K^b-positive Rag2$^{-/-}$ splenocytes, in terms of frequency (Fig. 1*G*, *Upper*) and total amount (Fig. 1*G*, *Lower*), compared with Des CD8 T cells from Des.KK^b mice. Furthermore, CD8 T cells from *Dkk3*$^{-/-}$.Des.KK^b mice showed increased expression of the activation markers CD25 and CD69 compared with CD8 T cells from Des.KK^b mice (Fig. S6). Taken together, DKK3 contributes to the hyporesponsiveness of CD8 T cells from tolerant Des.KK^b 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, K^b-positive, carboxyfluorescein succinimidyl ester (CFSE) labeled target cells were eliminated equally well in both mouse groups (Fig. 2*A*). 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. 2*B*) or with OVA-expressing adenovirus (Fig. 2*C*). Wild-type and *Dkk3*$^{-/-}$ mice developed comparable reactivity in both cases.

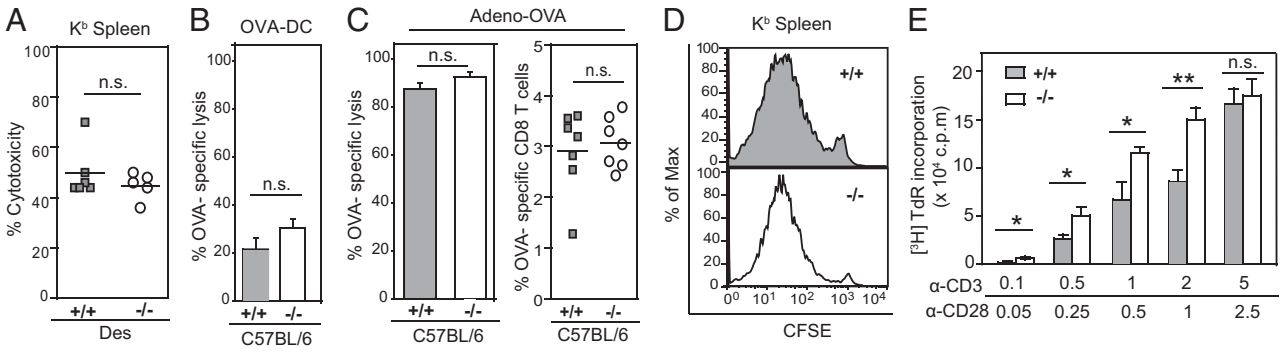

Fig. 2. Hyperresponsiveness of DKK3-deficient T cells does not account for the loss of tolerance in *Dkk3*$^{-/-}$.Des.KK^{b} mice. (*A*) Des and *Dkk3*$^{-/-}$.Des mice were immunized with activated H-2^{b} dendritic cells. Comparable cytotoxicity in vivo was detected by quantification of CFSE^{high} K^{b}-positive splenocytes 7 d after immunization. Data are pooled from three independent experiments (n.s.: Des $n = 5$, *Dkk3*$^{-/-}$.Des $n = 7$) (*B*) Wild-type and *Dkk3*$^{-/-}$ C57BL/6 mice were immunized with OVA-loaded dendritic cells (OVA-DC), and cytotoxicity was measured 15 d later by quantification of CFSE-labeled OVA-loaded splenocytes. The cytoxoxicity levels (mean ± SEM) representative of four independent experiments are shown (n.s.: $P = 0.2$; $n = 4$–8 mice per group). (*C*) Wild-type and *Dkk3*$^{-/-}$ C57BL/6 mice were immunized with recombinant adenovirus-expressing OVA (Adeno-Ova). The percentage of OVA-specific lysis in vivo (*Left*) and the percentage of OVA-specific CD8 T cells (*Right*) was measured at day 28 after immunization. Representative data of one of two experiments are shown. Cytoxoxicity levels (mean ± SEM; n.s.: $P = 0.1$; $n = 7$ mice per group), percentage of OVA-specific CD8 T cells determined by K^{b}-OVA pentamer staining (mean ± SEM; $P = 0.7$; $n = 7$). (*D*) Proliferation of purified CFSE-labeled CD8 T cells from Des and *Dkk3*$^{-/-}$.Des mice observed 2 d after stimulation with 10^5 irradiated splenocytes from *Rag2*$^{-/-}$ mice in vitro. Representative data from three separate experiments are depicted ($P = 0.16$; $n = 3$). (*E*) T cells from *Dkk3*$^{-/-}$ and *Dkk3*$^{+/+}$ C57BL/6 mice were stimulated with titrated concentrations of anti-CD3ε and anti-CD28 antibodies. Proliferation was determined at day 3. Representative thymidine incorporation (c.p.m, mean ± SEM) of one of four independent experiments is shown (**$P < 0.01$, *$P < 0.05$).

Moreover, lack of DKK3 did not influence the expansion of OVA-specific CD8 T cells in response to OVA-expressing adenovirus (Fig. 2*C*). 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*$^{-/-}$.Des mice were stimulated in vitro with irradiated *Rag2*$^{-/-}$ splenocytes (Fig. 2*D*), 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. 2*E*). 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 K^{b}-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. 3*A*). 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. S7). Des CD8 T cells, stained with CFSE and activated against the K^{b} antigen, showed an impaired proliferative capacity in the presence of DKK3-secreting astrocytes (Fig. 3*B*). This inhibitory effect was reversed by addition of a blocking DKK3-specific antibody (Fig. 3*B*). 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 K^{b} stimulation in comparison with supernatant of untransfected HEK 293T cells (Fig. 3*C*). 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.KK^{b} 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

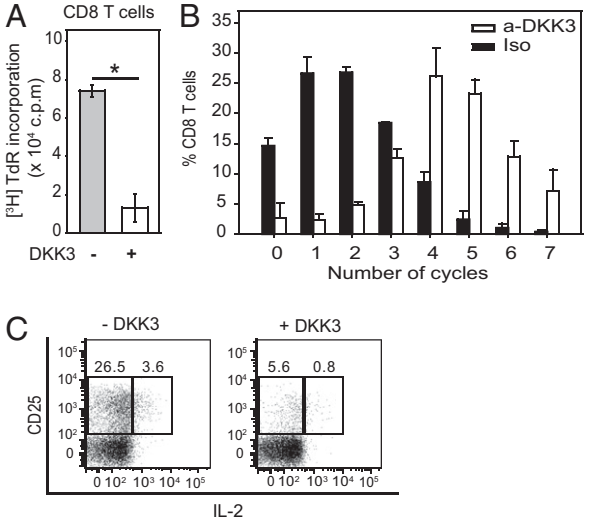

Fig. 3. Exogenous DKK3 inhibits CD8 T-cell responses in vitro. (*A*) Purified CD8 T cells were cocultured with irradiated (30 Gray) control and *Dkk3*-transfected HEK 293T cells in the presence of anti-CD3ε (2 μg/mL) and anti-CD28 (1μg/mL). T-cell proliferation was assessed by thymidine incorporation 3 d after stimulation (c.p.m, mean ± SEM). Data from one of three independent experiments are shown (*$P < 0.05$). (*B*) Purified CFSE-labeled CD8 T cells from Des mice were stimulated with 10^5 irradiated Rag2$^{-/-}$ splenocytes in the presence of astrocytes. *Dkk3*-blocking (5 μg/mL) or control antibodies (5 μg/mL) were added to the cultures, and proliferation of CD8 T cells was measured at day 3. Cycle numbers (mean ± SEM) of two independent experiments are shown ($n = 3$). (*C*) Purified T cells from *Dkk3*$^{-/-}$. Des mice were stimulated with 10^5 irradiated splenocytes from *Rag2*$^{-/-}$ mice in the presence of supernatant of *Dkk3*-transfected HEK 293T cells or supernatant of untransfected HEK 293 cells (1:2 dilution). CD25 expression and IL-2 production were measured 1 d later by flow cytometry. Representative dot plots of gated CD8 T cells (isotype control antibody staining for CD25 and IL-2 antibodies <0.5%) of three independent experiments are shown.

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eliminate K^b-positive tumors (15) and to kill K^b-positive target cells. DKK3-sufficient or -deficient Des.KK^b mice and control Des mice were injected s.c. with P815.K^b.B7 tumor cells. The majority of tolerant Des.KK^b mice accepted the tumor cells, whereas Des mice readily rejected them (Fig. 4 *A* and *B*). *Dkk3*$^{-/-}$.Des.KK^b mice were not tolerant, as they rejected the P815.K^b.B7 tumor graft with similar efficiency as Des mice (Fig. 4 *A* and *B*). As shown in Fig. 2*A*, Des CD8 cytotoxic effector T cells are induced in Des mice upon stimulation with activated K^b-positive C57BL/6 dendritic cells. By contrast, reduced K^b-specific cytotoxicity was observed in tolerant Des.KK^b mice (Fig. 4*C*). Deletion of *Dkk3* resulted in restoration of K^b-specific cytotoxicity in the Des.KK^b mice (Fig. 4*C*). Thus, DKK3 function is required for CD8 T-cell tolerance in Des.KK^b mice.

Blockade of DKK3 Reverses CD8 T-Cell Tolerance in Vivo. CD8 T-cell tolerance in Des.KK^b mice is based, at least in part, on the capacity of neonatally induced tolerant Des CD8 T cells to regulate naive Des CD8 T cells that left the thymus at a time when the K^b antigen in the skin was no longer accessible (15). To clarify whether DKK3 is crucial for the maintenance of CD8 T-cell tolerance during adulthood, we blocked its function by administering neutralizing DKK3-specific antibodies to adult tolerant Des.KK^b mice and then assessed CD8 T-cell reactivity against autologous skin grafts. Des CD8 T cells from single transgenic Des mice are capable of rejecting K^b-positive skin grafts, whereas tolerant Des.KK^b mice accept such grafts (13). Therefore, we assumed that activated Des CD8 T cells may infiltrate and reject the K^b-positive autologous skin graft in the absence of T-cell tolerance. Indeed, blockade of DKK3 function by the neutralizing antibody abrogated tolerance and resulted in rejection of the K^b-positive autologous skin graft (Fig. 4*D*) in Des.KK^b mice. The treated Des.KK^b mice did not develop a general autoreactivity in the skin. This observation may be explained by the finding that activated CD8 T cells migrate preferentially to inflamed but not to healthy skin (22). The majority of control mice treated with isotype control IgG accepted the transplanted autologous skin (Fig. 4*D*). The DKK3-specific antibody did not prevent autologous skin graft acceptance in CBA/J mice (Fig. S8). CBA/J mice harbor low frequency of skin-specific CD8 T cells, unlike Des.KK^b mice, in which the peripheral repertoire is strongly skewed toward the K^b 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.

DKK3-Producing Tolerant Des CD8 T Cells Are Sufficient to Induce Tolerance. So far we have shown that DKK3 is indispensable for CD8 T-cell tolerance. To assess whether DKK3 produced by tolerant Des CD8 T cells per se is capable of mediating tolerance, we performed adoptive T-cell transfer experiments. Purified Des CD8 T cells from tolerant Des.KK^b.*Rag2*$^{-/-}$ mice were transferred into nontolerant *Dkk3*$^{-/-}$.Des.KK^b mice (Fig. 5*A*). Before transfer, CD4 and CD8 T cells were depleted in the recipients to allow for sufficient numbers of antigen-inexperienced recent thymic emigrants to be targeted for regulation. One day after transfer, recipient mice received P815.K^b.B7 tumor cells. Control *Dkk3*$^{-/-}$.Des.KK^b mice were treated in the same way and received purified CD8 T cells that were specific for the SV40 large T antigen [TCR 8, which does not cross-react with the K^b antigen (15, 23)]. P815.K^b.B7 tumors were rejected in these control mice due to activation of DKK3-deficient T cells that remain after depletion and/or new thymic emigrants (Fig. 5 *B* and *C*). By contrast, tumor growth was seen in most of the *Dkk3*$^{-/-}$.Des.KK^b mice that had received the tolerant Des CD8 T-cell population (Fig. 5 *B* and *C*). This indicates that *Dkk3*$^{-/-}$ T cells are suppressed by the transferred tolerant Des CD8 T cells. Furthermore, blockade of DKK3 function by the neutralizing DKK3-specific antibody resulted in P815.K^b.B7 tumor rejection by *Dkk3*$^{-/-}$Des.KK^b mice that had received tolerant Des CD8 T cells (Fig. 5*D*). Thus, *Dkk3* produced by Des CD8 T cells is sufficient to mediate peripheral CD8 T-cell tolerance in a *Dkk3*$^{-/-}$ host.

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 in vivo CD8 T-cell tolerance; and (*v*) DKK3-producing tolerant CD8 T cells can regulate *Dkk3*$^{-/-}$ CD8 T cells with the same antigen specificity in an 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

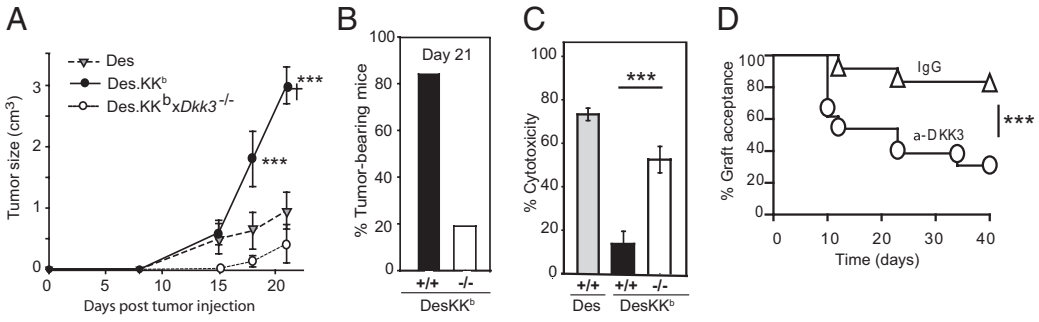

Fig. 4. *Dkk3* is indispensable for CD8 T-cell tolerance in vivo. (*A*) Des ($n = 23$), Des.KK^b ($n = 19$), and *Dkk3*$^{-/-}$.Des.KK^b ($n = 21$) mice were inoculated s.c. with 2×10^5 DKK3-negative P815.K^b.B7 tumor cells. Tumor sizes (mean ± SEM) pooled from two independent experiments ($P < 0.0001$, Mann–Whitney *U* test) are shown. (*B*) The percentage of Des.KK^b and *Dkk3*$^{-/-}$. Des.KK^b tumor-bearing mice at day 21 is shown ($P < 0.05$, Fisher's exact test). (*C*) Des, Des.KK^b, and *Dkk3*$^{-/-}$.Des.KK^b mice were immunized with activated H-2^b dendritic cells, and the percentage of lysis in vivo of CFSE^{high} K^b-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.KK^b 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).

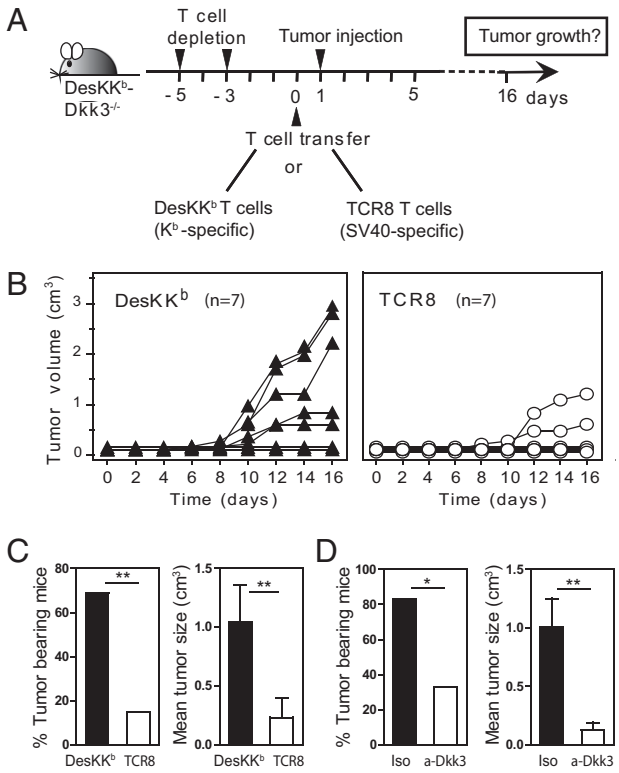

Fig. 5. DKK3-producing tolerant Des CD8 T cells are sufficient to mediate tolerance. (*A*) *Dkk3*$^{-/-}$. Des.KK^b mice were treated on days –5 and –3 with 0.5 mg anti-CD4 (GK1.5) and 0.5 mg anti-CD8 (53.6.7) depleting antibodies. At the time of T-cell transfer (day 0) less than 10% of the original CD4 and CD8 T cells was left in the blood of the treated mice. Purified CD8 T cells (10^5) either from day 12 thymectomized Des.KK^b.*Rag2*$^{-/-}$ mice or from day 12 TCR8 mice were transferred. One day later, mice were inoculated s.c. with 2×10^5 P815. K^b.B7 tumor cells. (*B*) Kinetics of tumor growth in recipients of Des.KK^b (*Left*) and TCR8 (*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 TCR8 and Des.KK^b recipients (mean ± 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 0 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 ± SEM, $P < 0.01$; $n = 12$).

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^b-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 into *Dkk3*$^{-/-}$.Des.KK^b mice after depleting the CD4 and CD8 T cells in the recipient mice. In this setting, the transferred Des CD8 T cells are the sole source of DKK3, and the DKK3-deficient T cells, mainly antigen-inexperienced, new thymic emigrants, are the only T cells capable of rejecting the K^b-positive tumor. As we observed tolerance in these mice and as we could abrogate this tolerance by the neutralizing anti-DKK3 antibody, we conclude that DKK3-deficient T cells can be regulated and that DKK3 produced by Des CD8 T cells is sufficient to induce peripheral CD8 T-cell tolerance in a *Dkk3*$^{-/-}$ host.

The essential contribution of CD8 regulatory T cells to peripheral tolerance is currently being evaluated with increasing interest (9–11, 28). Peripherally induced regulatory CD8 T subsets can originate from T-cell populations without any initial regulatory capacity. They can acquire their suppressive activity under various conditions, such as by alterations in costimulatory molecule interactions (29) or following antigen recognition by unique antigen-presenting cells in so-called immune-privileged sites, such as B cells in the anterior chamber (30) or keratinocytes in hair follicles in our model. As shown here for the tolerant Des CD8 T cells, CD8 regulatory T cells display their activity mostly in an antigen-specific way (10). No specific markers have been identified so far for regulatory CD8 T cells (31). Therefore, it will be interesting to test whether DKK3 is a mediator of the CD8 T-cell suppressive activity in other models of CD8 T-cell regulation.

Our work opens perspectives to evaluate the involvement of DKK3 in tissue-induced T-cell tolerance and autoimmunity. As DKK3 is preferentially expressed in organs classically termed "immune privileged" (20, 21), it will be interesting to clarify whether or not DKK3 may contribute to the immune-suppressive milieu of such tissues.

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 CBA background and then intercrossed to homozygocity 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^{dxk} mice are syngeneic for cells from CBA.K^b mice and for the P815.K^b.B7 tumor except for the K^b antigen. C57BL/6 mice and CBA/J 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 Regierungspräsidium (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 ×63-Ag8.653. Hybridomas were screened by ELISA for reactivity with a

DKK3-GST fusion protein. Hybridoma *Dkk3*-4.22 (IgG1 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 seperated 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 Substrate (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).

T-Cell Proliferation and IL-2 Assays. Lymphocytes (10^5) were cultured in 96-well plates coated with anti-CD3ε 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 (Biolegend). In the coculture assays, 25×10^3 of either HEK 293T cells or primary astrocytes were plated in 96-well plates. Where indicated, anti–*Dkk3*-4.22 (10 μg/mL) or isotype control (10 μg/mL) antibodies were added to the medium. At day 1, 10^5 purified CFSE-labeled Des CD8 T cells and 10^5 irradiated $Rag2^{-/-}$ splenocytes were added to the plate. Proliferation was measured at the indicated time points.

Primary Astrocyte Cultures. Brain cell suspension (32) was plated in 75-cm^2 cell culture flasks (Nunc), precoated with poly-L-lysine (20 μg/mL; Sigma). At day 14, the cells were replated on bacteria-grade dishes (Sarstedt). The astrocyte-containing supernatant was transferred to cell culture grade dishes (Nunc).

Cytotoxic T lymphocyte (CTL) Mediated Cytotoxicity in Vivo. Mice were immunized with 1×10^6 bone marrow-derived dendritic cells from C57BL/6 mice that had been activated with 0.5 μM CPG-ODN 1668 in case of K^b-specific responses. For OVA-specific immunization, dendritic cells were loaded with 1 μmol SIINFEKL peptide. Alternatively, mice were immunized with 10^8 infectious units of adenovirus-expressing OVA. Adenoviral stocks were grown and purified as described (33). CTL activity in vivo was quantified against target cells expressing K^b or loaded with SIINFEKL peptide 7 d after immunization as described (15). For tetramer staining, single-cell suspensions of spleens were incubated with phycoerythrin-labeled K^b/SIINFEKL tetramers (ProImmune) for 20 min.

Statistical Analyses. Statistical analyses were performed by using 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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