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DAP10 Deficiency Breaks the Immune Tolerance against Transplantable Syngeneic Melanoma

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DAP10, an activating adaptor protein, associates with the NKG2D protein to form a multisubunit receptor complex that is expressed in lymphoid and myeloid cells. The ligands for NKG2D-DAP10 receptor are expressed in both normal and tumor cells, suggesting distinct roles for this receptor in autoimmunity and cancer. In this study, we report that constitutive DAP10 activating signaling is part of regulatory mechanisms that control immunity against tumors. Mice lacking DAP10 (DAP10KO), showed enhanced immunity against melanoma malignancies due to hyperactive functioning of NK1.1<sup>+</sup>CD3<sup>+</sup> NK cells. DAP10 deficiency resulted in substantially increased NKT cell functions, including cytokine production and cytotoxicity, leading to efficient killing of melanoma tumors. Moreover, the antitumor phenotype of DAP10KO mice correlated with impaired activation status of CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells (Tregs). Upon activation, DAP10KO Tregs maintained higher levels of IL-2 and produced significantly lower amounts of IL-10 and IFN-γ cytokines when compared with wild-type Tregs. Our data suggest that DAP10 signaling is involved in adjusting the activation threshold and generation of NKT cells and Tregs to avoid autoreactivity, but also modulates antitumor mechanisms. The Journal of Immunology, 2007, 179: 3763–3771.

The activation threshold of immune cells is regulated by activating and inhibitory signals received through recognition of self and foreign Ags. Genetic defects that affect activating or inhibitory receptors renders the immune system unable to distinguish between self and non-self causing autoimmunity or abnormal response against infectious agents and transformed cells (1, 2). Many activating receptors are multisubunit complexes in which the transmembrane adaptor proteins are responsible for transducing signals inside the cell. DAP10 is a transmembrane adaptor protein that associates with the activating receptor NKG2D in a multisubunit receptor complex expressed on hemopoietic cells (3). NKG2D-DAP10 receptor complex is expressed constitutively on murine and human NK cells, γδ T cells, and NKT cells, and innate stimuli can further up-regulate its expression (3–6). Upon activation, murine CD8<sup>+</sup> T cells express NKG2D-DAP10 receptor that participates in regulation of adaptive immune response (7, 8). The expression of NKG2D at the cell surface requires its association with DAP10 protein. This association involves interaction between an acidic amino acid in the transmembrane region of DAP10 and a basic amino acid in the transmembrane domain of NKG2D protein (3). The expression patterns of NKG2D and DAP10 do not completely overlap, so it is possible that DAP10 associates with other yet unidentified receptors, especially in some myeloid cell populations. In humans, NKG2D associates exclusively with DAP10 (3, 4), whereas mice express two different isoforms for NKG2D, a long form, which associates only with DAP10, and a short form, which has been shown to pair with both DAP10 and DAP12 (9, 10).

Unlike DAP12 and other adaptor proteins, DAP10 does not signal via the ITAM, but contains an YXXM motif involved in activation of PI3K pathway (3). The association of DAP10 with the p85 regulatory subunit of PI3K is followed by binding to Grb2 and Vav1 signaling proteins, triggering activation signals inside the cell (3, 11, 12). In NK cells, DAP10 signaling directly induces cytotoxicity and enhances cytokine production initiated via DAP12-associated receptors (11, 13). In T cells, it provides primarily costimulation for TCR-induced signals (5).

The identified ligands for NKG2D-DAP10 receptor complex are MHC class I-like proteins, including MHC class I-related chain A (MICA), MICB, and UL16 binding proteins in human and RAE-1, H60, and MULT-1 in rodents (4, 14–17). In general, they are minimally expressed in adult tissues and are up-regulated in tumor cells and pathogen-infected cells. Because NKG2D ligands are tumor-associated Ags, NKG2D-DAP10 receptor complex is hypothesized to play a role in immune surveillance against tumors. It is now well established that expression of NKG2D ligands in tumor cells results in rejection of tumors from the host (18–20). NKG2D ligands, however, can be expressed in normal tissues. For example, H60 Ag (an NKG2D ligand) is expressed on resting myeloid cells, which can directly stimulate H60-specific T cells responses (21). RAE-1 molecules are also highly expressed in embryonic tissues and induced upon activation in myeloid cells (14, 22). Thus, the expression pattern of NKG2D-DAP10 receptor complex and its ligands suggest that they are involved in immune recognition in the context of both self and abnormal self.

In this study, we investigate the physiological role of DAP10 and its importance in tumor immunity by analyzing the DAP10KO mice. Although it is generally believed that DAP10
plays a role in tumor immunosurveillance, we unexpectedly observed that DAP10 deficiency enhanced immunity against melanoma malignancies by affecting the regulatory functions of Tregs and activating the antitumor effector functions of NK1.1 cells.

Materials and Methods

Mice

All experimental mice lacking DAP10 (DAP10KO mice), which were generated at DNAX, and control wild-type (WT) C57BL/6J mice from The Jackson Laboratory were used at 6–10 wk of age and matched by sex and age. Mice were housed in a pathogen-free animal facility. All animal procedures used in this study were approved by DNAX Institutional Animal Care and Use Committee.

B16 melanoma tumor model

For induction of B16 pulmonary metastases, B16 melanoma cells (American Type Culture Collection (ATCC)) were harvested for injection when in the logarithmic growth phase (±50% confluent). WT and DAP10KO C57BL6 mice were injected i.v. with 1 × 10⁶ B16 cells or as indicated in each experiment. Lungs were removed 2 wk after tumor inoculation, and metastases were counted using a dissecting microscope.

Mice were depleted of NK1.1+ cells using anti-NK1.1 mAb clone PK136 (ATCC) at 0.5 mg per mouse, injected i.v. on day −1 and then every 3 days. Mouse IgG2a isotype control Ab clone PAB 350 (ATCC) was used for the control groups. Anti-CD25 mAb clone PC-61 (ATCC) was used for the control groups. Anti-CD25 mAb clone PC-61 (ATCC) was injected i.v. at 0.5 mg per mouse to deplete T regulatory cells (Tregs), at the indicated days for each experiment. To deplete NK cells, mice were injected i.v. with 30 μl of anti-asialo-GM1 Ab (Cederlane Laboratories) at day −2 and then every 4 days. All Abs used for in vivo studies were endotoxin-free. The efficiency of in vivo depletions was verified by FACS analysis of splenic leukocytes. Anti-NK1.1 treatment resulted in depletion of >95% of the NK1.1+ cells, whereas asialo-GM1 treatment depleted >85% of NK cells without affecting the percentages of NKT cells.

Generation of bone marrow chimeras

Recipient mice were exposed to one dose of 1000 rad of gamma irradiation. Bone marrow cells were isolated from donor mice and 1.5 × 10⁶ cells were delivered i.v. into the tail vein of the recipient mice. At 6 and 8 wk after reconstitution, peripheral blood cells, and splen cells of recipient mice were analyzed by flow cytometry to assess the levels of reconstitution. WT mice used in this experiment were GFP-positive, which were a gift of Dr. B. Schaefer (Uniformed Services University of the Health Sciences, Bethesda, MD). At 8 wk, 95–98% of recipient cells were derived from donors. Viability of bone marrow chimeras was 100%.

TagMan analysis

Total RNA was isolated from lymph nodes, spleens, thymuses, and lungs of WT BL/6 and DAP10KO mice and subjected to TagMan analysis. Primers for all genes were designed using Primer Express (Applied Biosystems), and selected for sequences that have no cross-reactivity with other family members.

In vitro functional analysis

NKT cells were sorted from B cell-depleted splenocytes (purity ≥95%). Cytokines were analyzed following activation of NKT cells with plate-bound anti-CD3 Ab (clone 17A2) or rat IgG2b (clone A95-1) isotype control (BD Pharmingen) in Iscove’s medium supplemented with 10% FCS, 100 mM penicillin, 100 mM streptomycin, 100 mM Glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 5.5 × 10⁻³ M 2-ME, and 25 mM HEPES. All culture reagents were from Invitrogen Life Technologies. Supernatants were collected after 48 h and cytokines were measured as described below.

Cytotoxicity assays were performed following expansion of NKT cells in complete Iscove’s medium containing 50 mg/ml mouse IL-2 (PeproTech). A standard 4-h ⁵¹Cr release assay was performed (23).

CD⁴⁺/CD⁵⁶⁺ Tregs were isolated from splenocytes using the Treg isolation kit of Miltenyi Biotec (purity ≥96%). A total of 2 × 10⁶ Tregs were cultured in the presence of IL-2 (20 ng/ml) or IL-2 and soluble anti-CD3 Ab (10 μg/ml) for 48 h. Supernatants were collected and cytokines were measured. For mRNA analysis, 3.5 × 10⁶ Tregs were activated with IL-2 (20 ng/ml) and plate-bound anti-CD3 Ab (5 μg/ml) for 6 h.

Pulmonary leukocytes were prepared by mincing lungs and incubating them with complete medium supplemented with collagenase (1 mg/ml) and DNase I (1 mg/ml), during 1 h at 37°C. The digested tissues were passed through a 75-μm nylon mesh. Red cells were lysed and cells were washed twice with PBS. Cells were resuspended in 6 ml of 40% Percoll (v/v) and layered onto 6 ml 80% Percoll (v/v), and then centrifuged at 600 × g, for 20 min at 15°C.

Flow cytometry and cytokine analysis

Mouse cells were phenotyped by flow cytometry after blocking Fe receptors with anti-CD16/CD32 Ab. Two- or three-color staining was performed using the following Abs: anti-CD3 FITC, anti-CD8 CyChrome (clone 17 A2), anti-CD4 FITC (clone GK1.5), anti-CD25 PE or anti-CD25 biotin Ab

FIGURE 1. Phenotype of naive WT and DAP10KO lymphoid organs. A. Spleens were removed from 8-wk-old naive mice (n = 3 per group) and weighted. Results are shown as mean ± SD. B. Splenocytes were isolated in 6- to 8-wk-old mice and counted after lysis of red cells. The differences are statistically significant (p = 0.04) as determined by one-tailed t test for n = 6 mice. C. Mice were injected i.v. with Brdu (1 mg per mouse). After 24 h, splenocytes isolated from WT and DAP10KO mice (n = 3 per group) were depleted in red cells and stained to detect the Brdu incorporation using a FITC Brdu kit. Stained cells were analyzed by flow cytometry. Results are presented as the percentages of splenocytes that incorporated Brdu and are representative of two independent experiments. Data are expressed as mean ± SD. The enhanced proliferation of DAP10KO cells compared with WT cells is statistically significant (p < 0.05), as determined by one-tailed, unpaired t test for n = 8 mice. D. RT-PCR analysis of splenocytes. Total RNA was isolated from spleens of WT and DAP10KO mice (n = 3 per group), pooled and subjected to real-time RT-PCR analysis using specific primers for an array of cytokines. mRNA levels were normalized to ubiquitin levels. E. Seric levels of cytokines from mice used in C were determined using a mouse inflammatory CBA kit.

1 Abbreviations used in this paper: WT, wild type; Treg, T regulatory cell.
clone PC61), anti-NK1.1 PE (clone PK136), IgG2a κ PE- or biotin-conjugated (clone 49.2), IgG1 PE- or biotin-conjugated (clone 107.3). Streptavidin-CyChrome was used to detect biotin labeling. All of the reagents were from BD Pharmingen. Anti-NKG2D PE (clone CX5) was a generous gift of Dr. L. Lanier (University of California, San Francisco, CA).

The in vivo proliferation rate of NKT cells was determined by measuring the incorporation of BrdU using the FITC BrdU flow kit (BD Pharmingen).

The BD Biosciences mouse Th1/Th2 or inflammatory CBA kits were used to measure the levels of cytokines produced by NKT cells or Tregs. Samples were analyzed on a FACSCalibur flow cytometer. CellQuest, BD Biosciences CBA, and FlowJo software products were used to analyze the acquired data. All contour plots were set at 5%.

Statistical analysis

All data are representative of at least two to three experiments. Statistical analysis was conducted by unpaired Student’s t test using GraphPad PRISM software. Differences were considered significant when for values of $p < 0.05$.

Results

DAP10 deficiency predisposes a hyperimmune environment

The generation of DAP10KO mice was performed by using a conventional gene targeting vector to remove exons 3 and 4 of DAP10 gene (24). Naive DAP10KO mice appeared to have normal organogenesis as assessed by histology. However, we observed that DAP10KO spleens had increased weights and cell numbers compared with WT and this was associated with increased proliferation rate of CD3+ and CD3− splenocytes (Fig. 1, A–C). Flow cytometry analysis of splenic leukocytes isolated from WT and DAP10KO naive mice ($n = 30$ per group) showed differences in the frequencies of CD4+ T cells: DAP10KO CD4+ T cells represent 16.4 ± 0.07% of splenic cells, whereas WT CD4+ T cells represent 13.2 ± 3.58% of the splenic population. Splenomegaly was particularly marked in DAP10KO mice induced to develop autoimmunity, and this correlated with enlarged red pulp zone in the spleen, an increased number of splenocytes, and increased proliferation of Ag-specific T cells (24).

The hyperactive phenotype of DAP10KO spleens cannot be due to a possible aberrant functioning of NKG2D protein as
constitutive expression of NKG2D is impaired in DAP10KO splenocytes (24).

**DAP10 deficiency causes NKT cell hyperactivity**

Flow cytometry analysis of splenic DAP10KO NKT cells revealed variable frequencies with some mice (21/28) demonstrating decreased percentages when compared with WT NKT cells (Fig. 2A, *top contour plots*) or (7/28) almost normal percentages (Fig. 2A, *bottom contour plots*). In addition, resting DAP10KO NKT cells that were sorted by flow cytometry using anti-NK1.1 and anti-CD3 Abs had reduced frequencies of NK1.1<sup>+</sup>CD4<sup>+</sup> cell subpopulation, when compared with resting WT NKT cells (data not shown). It has been reported that in vivo activation of NKT cells is associated with their rapid depletion from peripheral lymphoid organs, followed with proliferation and repopulation from bone marrow-derived cells (25). Based on this knowledge, we analyzed the proliferative rate of splenic and bone marrow NKT cells in WT and DAP10KO mice. As shown in Fig. 2B, DAP10KO NKT cells had a significantly increased turnover proliferation rate compared with WT cells, suggesting that they are constitutively hyperactive.

We then assessed whether DAP10 deficiency can affect the capacity of NKT cells to produce cytokines. Because NKT cells were purified using anti-CD3 Ab, which may slightly activate NKT cells, we could not assess the function of truly resting NKT cells. Purified NKT cells cultured with IgG isotype control Ab (Fig. 2C, resting), produced low levels of cytokines. Stimulation of NKT cells with plate-bound anti-CD3 Ab induced the production of all NKT signature cytokines, including IL-4, IFN-γ, TNF-α, and IL-2 (Fig. 2C, anti-CD3 activated). Interestingly both unstimulated and stimulated DAP10KO NKT cells produced significantly higher amounts of cytokines when compared with WT cells. These results suggest that DAP10 signaling is required for normal NKT cell functions, whereas loss of DAP10 results in a constitutive hyperactivity of tissue NKT cells.

**DAP10 deficiency confers protection to B16 melanoma metastases, and NKT cells are the effectors responsible for the antitumor phenotype**

Because activation of NKT cells has been shown to be critical in prevention of metastases of B16 melanoma tumors (26), we wondered whether the constitutively activated NKT cells observed in DAP10KO could influence tumor immunity against B16 melanoma metastases. We induced pulmonary metastases by injecting 10<sup>5</sup> B16 cells i.v. at various concentrations into WT and DAP10KO mice. Two weeks later, lungs were removed and analyzed for
metastatic colonies. As shown in Fig. 3, A and B, DAP10KO mice injected with $1 \times 10^5$ B16 cells developed three to five times fewer pulmonary metastases than WT animals. DAP10KO mice injected with $1 \times 10^5$ B16 cells were completely protected from melanoma metastases (Fig. 3A).

To investigate the hemopoietic origin of the antitumor activity in DAP10KO mice, we established stable bone marrow chimera. Irradiated DAP10KO mice reconstituted with WT bone marrow cells developed B16 metastases at similar frequencies to those of WT animals (Fig. 3C). In contrast, reconstitution of WT mice with DAP10KO bone marrow cells resulted in resistance to metastases comparable to that observed in DAP10KO animals. These results clearly demonstrate that hemopoietic cells are responsible for the DAP10KO antitumor activity.

TagMan analysis of lungs harvested from mice 2 wk after tumor injection (Fig. 3D) displayed a significant increase in mRNA levels for IFN-γ and IL-12 cytokines in DAP10KO as compared with WT mice. Interestingly, both IFN-γ and IL-12 have been shown to be involved in efficient eradication of melanoma tumors (26–28). Flow cytometry analysis of pulmonary leukocytes showed twice higher infiltration of pulmonary DAP10KO NK1.1+ cells, as well as enhanced intracellular IFN-γ production by DAP10KO pulmonary leukocytes (Fig. 3E).

Because both NK and NKT cells can mediate potent in vivo rejection of B16 tumors (26, 28), in vivo depletion studies were performed to delineate the involvement of these cells in the DAP10KO enhanced antitumor activity against B16 metastases. Depletion of NK1.1+ cells, which includes both NK and NKT cells, from WT animals resulted in a small but significant increase in the number of lung B16 metastases as compared with untreated WT animals (Fig. 4A). In addition, WT mice treated with anti-NK1.1 mAb presented B16 metastases in organs other than lungs, such as liver, skin, abdominal, and thoracic cavity, indicating that NK1.1+ cells in WT animals confer some minor degree of incomplete protection from B16 melanoma metastases. Interestingly, depletion of NK1.1+ cells from DAP10KO mice resulted in complete loss of antitumor activity with lungs displaying B16 metastases equivalent to WT animals. To distinguish between NK and NKT cell functions in vivo, mice were treated with anti-asialo-GM1 Ab to selectively deplete NK cells (29). In contrast to the NK1.1 depletions, removal of NK cells had essentially no effects on the antitumor activity of DAP10KO mice (Fig. 4B). The anti-asialo-GM1 Ab used in these depletion experiments was biologically active as it increased the susceptibility of both WT and DAP10KO mice to transplanted carcinoma tumors (24). These results clearly suggest that NKT cells are the major effector population responsible for the defense against B16 melanoma metastases in DAP10KO mice in vivo.

We next tested the in vitro cytotoxic capacity of resting or IL-2-activated NK and NKT cells to kill B16 tumor cells. Lysis of YAC-1 tumor targets that express NKG2D ligands was used as a positive control for NKG2D-dependent killing mechanisms. Resting naive WT or DAP10KO NK cells and NKT cells did not kill B16 tumor cells in vitro (data not shown). IL-2-activated DAP10KO NKT cells killed less efficiently NKG2D ligand expressing YAC-1 tumor cells when compared with WT NKT cells, and anti-NKG2D Ab partially inhibited the killing in both cases (Fig. 4D). These results suggest that activated DAP10KO NKT cells express a partially functional NKG2D receptor, although the expression level for NKG2D was extremely low to undetectable compared with WT cells (Fig. 4C). Interestingly, B16 melanoma cells were efficiently killed by DAP10KO NKT cells but not by WT NK cells, and this cytotoxicity was not affected significantly by anti-NKG2D Ab (Fig. 4D). These results are consistent with the in vivo data and indicate that DAP10KO NKT cells are programmed to efficiently kill these syngeneic tumor cells and that they can mediate efficient rejection of B16 melanoma tumors in vivo.
ENHANCED ANTITUMOR IMMUNITY IN DAP10KO MICE

To further understand the mechanistic pathways enabling DAP10-deficient mice to mount a better antitumor response, we wanted to determine whether DAP10 deficiency affects the immune environment that controls NKT cell functions. Several studies have shown that Tregs inhibit the immune response against self, and that elimination of Tregs in mice leads to efficient rejection of syngeneic tumors, including B16 melanoma (30, 31). In addition, it has been shown that human Tregs can suppress certain NKT cell activities, including cytokine production and cytotoxicity (32). Consequently, we hypothesized that DAP10 deficiency results in dysfunctional Tregs that are unable to suppress the activities of NKT cells, thereby leading to enhanced tumor rejection. We tested this hypothesis by analyzing B16 melanoma metastases in mice depleted of CD25+ Tregs. WT mice depleted of Tregs by the in vivo anti-CD25 Ab treatment showed remarkably decreased melanoma metastases similar to what was observed in untreated DAP10KO mice (Fig. 5A). DAP10KO mice depleted in CD25+ Tregs were completely free of tumors, demonstrating that DAP10KO Tregs have some suppressive activities in DAP10KO mice. Interestingly, WT NKT cells isolated from Treg-depleted mice were now capable of potent in vitro cytolytic activity of B16 melanoma cells, comparable to the cytotoxicity mediated by DAP10KO NKT cells (Fig. 5B). Again, in vitro killing of B16 tumors was NKGD2-dependent. Depletion of Tregs from WT mice had little effect on the ability of NKT cells to efficiently lyse YAC-1 tumor cells and this lysis was partially inhibited by anti-NKGD2 Ab, as was observed in WT mice (Figs. 4D and 5B).

To determine whether DAP10KO Tregs were dysfunctional in vivo, we transferred WT Tregs into DAP10KO mice and analyzed their impact on the development of B16 melanoma metastases. A total of 1 × 10⁶ WT Tregs were transferred i.v. into DAP10KO mice and 4 days later mice received B16 melanoma tumor cells. Control mice, both WT and DAP10KO received only B16 cells. Fig. 5C shows that transfer of WT Tregs in DAP10KO mice rendered them as susceptible to melanoma metastases as WT mice. Clearly, WT Tregs were capable of efficiently inhibiting the constitutive antitumor activity of DAP10KO mice.

Phenotypic analysis of freshly isolated Tregs from WT and DAP10KO mice showed that DAP10KO Tregs displayed a higher proportion of cells with low surface density CD25 (Fig. 6A). Interestingly, a subset of WT CD4+CD25low population expressed NKGD2 receptor and as expected this expression was absent in DAP10KO Tregs. The surface expression of other Ags, including CD28, CTLA-4, and CD62L, was present and unchanged in both CD25high and CD25low WT and DAP10KO Treg populations (data not shown). To further dissect the molecular differences in Tregs from WT and DAP10KO mice, TaqMan analysis of Tregs purified from naive splenocytes was performed. Despite the lack of DAP10 mRNA, Tregs from DAP10KO showed strong expression of NKGD2 mRNA, and equivalent expression levels of DAP12 and Foxp3 mRNAs (Fig. 6B). Recently, it was reported that Tregs with low surface expression of CD25 constitutively secrete IL-2 (33). IL-2 in conjunction with TCR signaling was shown to be critical for Treg proliferation and the development of suppressive functions (34). We next compared the levels of IL-2 transcripts between WT and DAP10KO Tregs at resting or activated conditions (Fig. 6C). Upon activation, WT and DAP10KO Tregs up-regulated Foxp3 transcripts ~4- and 3-fold, respectively. Interestingly, this up-regulation of Foxp3 was inversely correlated with the expression of IL-2. Activated DAP10KO Tregs appear to maintain higher levels of IL-2 transcripts. This response is probably due to increased frequencies of the CD4+CD25low population. We analyzed the production of IL-2 by WT or DAP10KO Tregs that were cultured with mitomycin C-treated splenocytes, acting as APCs, and activated or not through TCR. As shown in Fig. 6D, DAP10KO Tregs that were activated through TCR produced higher amounts of IL-2 than WT cells, whereas both WT and DAP10KO Tregs produced similar amounts of IL-2 when cultured only with APCs. Furthermore, DAP10KO Tregs activated with IL-2, or IL-2 plus soluble anti-CD3 Ab, produced lower amounts of IL-10 and IFN-γ when compared with WT cells (Fig. 6E).

**FIGURE 5.** DAP10 deficiency is associated with impaired Treg-mediated suppression. A, Development of metastases in mice depleted in Tregs. Mice were injected i.v. with anti-CD25 Ab (clone PC61, 0.5 mg per mouse), at days −3, 2, and 7, to deplete them in Tregs. Control mice were injected with isotype control (0.5 mg/mouse). Mouse (n = 5 per group) were injected with 1 × 10⁶ B16 cells i.v. at day 0, and metastases were counted at 2 wk. The differences between mice treated with anti-CD25 Ab and mice treated with isotype control were statistically significant (p < 0.05) for n = 10. B, The cytotoxic capacities of NKT cells isolated from mice depleted in Tregs. WT and DAP10KO mice (n = 5 per group) were depleted of CD25+ cells by i.v. injection of anti-CD25 Ab (clone PC61, 0.5 mg per mouse) at day −3. At day 0, NKT cells were isolated from spleens and cultured with mouse IL-2 for 7 days. NKT cells were then used as effectors in a cytotoxicity assay against YAC-1 and B16 melanoma targets. The data are representative of two experiments and are shown as mean ± SD. C, Adoptive transfer of WT Tregs in DAP10KO mice. DAP10KO mice were injected i.v. with 1 × 10⁶ WT Tregs or PBS at day −3. Mice were injected i.v. with B16 melanoma at day 0, and pulmonary metastases analyzed at day 14. The differences between DAP10KO mice that did or did not receive WT Tregs were statistically significant (p < 0.05) n = 10 mice per group.
Discussion

Because the NKG2D-DAP10 receptor complex is a potent activating receptors for NK cells and because NKG2D ligands are overexpressed on a variety of tumor cells, it has been hypothesized that the NKG2D-DAP10 complex is an important component of immune surveillance against tumors (35, 36). In undertaking these experiments, we logically predicted that DAP10KO mice should display impaired functioning of the NKG2D receptor leading to increased susceptibility to tumor growth and tumorigenic events. Interestingly, however, we did not observe any increase in the incidence of spontaneous tumors in DAP10KO mice, and DAP10KO mice were resistant to the development of carcinogen-induced skin tumors (24). Although the constitutive expression of NKG2D was impaired in DAP10KO lymphoid cells, the inducible surface expression was still present in short-term IL-2-activated DAP10KO NK cells. This result is in agreement with a previous study showing that the NKG2D-short form, which associates with DAP12, can be expressed as a functional receptor in activated DAP10KO NK cells (9). Thus, it is possible that NKG2D-DAP12 signaling might compensate for DAP10 deficiency in activated NK cells, which in turn may confer some protection against spontaneous tumors expressing NKG2D ligands. A recent study, however, showed that in vivo normal functioning of NKG2D on NK cells is impaired in DAP10KO mice (37).

Unexpectedly, DAP10KO mice also displayed enhanced antitumor immunity to experimentally induced B16 melanoma malignancies. B16 melanoma tumor cells express low levels of MHC class I and do not express known NKG2D ligands. Most of the melanoma Ags recognized by the immune system are nonmutated self-proteins, which distinguish different stages of melanocyte differentiation (38). These Ags are expressed by both normal and malignant melanocytes, explaining why they are tolerated by the immune system. Consequently, induction of self-reactivity to melanoma Ags is usually associated with tumor rejection (30, 39–41). In accord with this knowledge, the antitumor phenotype of DAP10KO mice was reversed either by the adoptive transfer of WT Tregs to DAP10KO mice, or by in vivo depletion of DAP10KO NKT cells. Both, Tregs and NKT cells are autoreactive cells that might well be involved in recognition of melanoma Ags. This possibility is supported by the identification of tumor-specific, nonmutated self-Ag as the physiological ligand for Tregs (42). In addition, in vivo activation of NKT cells was shown to inhibit melanoma metastases (26). It is conceivable that in WT mice, melanoma-associated Ags are recognized by Tregs and trigger their activation and subsequent inhibition of NKT cell functions. Indeed, in vivo depletion of WT mice in Tregs resulted in substantially decreased lung metastases, similarly to DAP10KO mice. In addition, WT NKT cells that were isolated from mice depleted in Tregs gained the capacity to kill B16 melanoma in vitro. By contrast, DAP10KO NKT cells were able to constitutively kill B16 melanoma, in vivo and in vitro, without elimination of Tregs. Likewise, restoring Treg functions in DAP10KO mice by infusion of WT Tregs inhibited DAP10KO
NKT antitumor activity to B16 melanoma tumor cells. These results suggest that DAP10KO Tregs are dysfunctional in their capacity to regulate endogenous NKT cell cytotoxic activity against syngeneic melanoma tumor cells. Consistent with this hypothesis, DAP10 deficiency was associated with constitutively hyperactive NKT cells. DAP10KO NKT cells showed increased proliferation rate in both spleen and bone marrow of naive mice. This increase was associated with decreased percentages of splenic DAP10KO NKT cells, which can be due to the down-regulation of NK1.1 and CD3 Ags in particular subsets of this population or to cell death, both phenomena characterizing NKT cell activation. In addition “resting” as well as activated DAP10KO NKT cells produced significantly higher levels of cytokines, including IFN-γ compared with WT NKT cells. This phenotype correlated with higher levels of IFN-γ transcripts in DAP10KO secondary lymphoid tissues and lungs. In vivo, higher early production of IFN-γ by NKT cells is usually followed by efficient tumor killing (27, 43). This pathway is likely to participate in NKT-mediated rejection of melanoma metastases in DAP10KO mice. 

Given that Tregs can also suppress the NK cell functions (44), it is possible that DAP10 deficiency can result in hyperactivation of NK cells. Indeed, anti-asialo-GM1 depletion studies have shown that NK cells are responsible for the resistance of DAP10KO mice to transplantable skin carcinoma tumors (24). Both, NKT cells and NK cells were shown to be involved in tumor eradication in this particular model (43). By contrast, impaired in vivo functioning of DAP10KO NK cells was observed in studies using the RMA leukemia model or a bone marrow transplantation model (24, 37). These results suggest that DAP10 may play distinct roles in different physiological processes. 

How does DAP10 signaling affect Treg functions? Phenotypic analysis of DAP10KO Tregs revealed an increased percentage of CD4+CD25low cells when compared with WT Tregs. DAP10KO Tregs that were stimulated via TCR and IL-2, maintained higher levels of IL-2 transcripts than WT Tregs. These data indicate that IL-2/IL-2R pathway is not functioning properly in DAP10KO Tregs. IL-2 is shown to be crucial for proliferation of Tregs and induction of proliferation results in loss of suppressive activities (45, 46). Interestingly, NKG2D was found to be expressed by a subset of CD4+CD25low Tregs in WT mice. A recent study reported that CD4+CD25low Tregs are self-reactive cells that produce IL-2 under physiological conditions. CD4+CD25low Tregs express low levels of Foxp3 and are not suppressive (33). Thus it is tempting to speculate that the DAP10 signaling pathway may control the transition of Tregs from a proliferative state to a functional suppressive status. By maintaining higher levels of IL-2 transcripts, DAP10KO Tregs expand but do not properly differentiate into mature suppressive cells. We propose that DAP10 signaling may be required for full activation of Tregs. Lack of this signaling weakens the relative suppressive status of Tregs, resulting in increased antitumor activity. It is well known that Tregs use two main mechanisms to suppress autoreactivity; the production of the immunosuppressive cytokines like IL-10, TGF-β, and IL-4 and the cell-cell contact interactions involving cell surface Ags such as CTLA-4 or glucocorticoid-induced TNFR (47, 48). IL-2-stimulation, with or without activation through TCR, resulted in significant reduced production of IL-10 by DAP10KO Tregs. The impaired production of suppressive cytokines by DAP10KO Tregs is likely to account for the decreased suppressive activities of those cells in vivo.

An important question raised by this study is whether DAP10 deficiency causes intrinsic effects in NKT cells, Tregs, or both. The defects in Tregs function could well be intrinsic, as suggested by the adoptive transfer of WT Tregs to DAP10KO mice. In addition, our preliminary data have shown reduced suppression exerted by DAP10KO Tregs on conventional CD4+ T cells functions, supporting the idea that DAP10 deficient Tregs are functionally impaired. Recently, the presence of suppressive NKG2D+CD4+ T cells was reported in human tumors (49). Taken together, these data suggest that NKG2D/DAP10 receptor complex can directly affect the suppressive functions of CD4+ Treg populations. In contrast, the effect of DAP10 deficiency on the hyperactive phenotype of DAP10KO NKT cells could be intrinsic as well, given that a small subset of these cells are CD25low.

Because DAP10 has immunoregulatory functions, blocking DAP10 may favor autoimmunity. However, naive DAP10KO mice did not develop spontaneous autoimmune reactions, nor was the rejection of tumors in these mice associated with an autoimmune phenotype. In particular, the resistance to melanoma metastasis was not associated with autoimmune vitiligo, therefore excluding a role of CD8+ T cells in this phenotype. Nevertheless, it will be interesting in the future to study the phenotype of DAP10KO mice that are induced to develop autoimmune diseases. Our data showed that the induction of the DTH response to a self-Ag, myelin oligodendrocyte glycoprotein peptide, (MOG35–55) triggered experimental autoimmune encephalomyelitis in 40% of DAP10KO mice but not in WT mice (24).

Besides NKT cells and Tregs, NKG2D-DAP10 receptor complex can be involved in differentiation of other autoreactive T cell populations. Two studies have shown an expression of the NKG2D-DAP10 receptor complex in autoreactive CD8+ and CD4+ T cells (7, 50). It will be of interest to understand whether DAP10 signaling affect the maturation of autoreactive T cells in the thymus or in the secondary lymphoid organs. NKG2D-DAP10 receptor complex is well expressed in the thymus, and the transcripts of NKG2D ligands were found to be highly expressed in the embryo (14). In addition, DAP10 signaling might influence the interaction between autoreactive T cells and APCs, which might be critical for the differentiation of autoreactive T cells in the periphery. DAP10 is expressed broadly in lymphoid as well as myeloid cells and DAP10 biology is therefore expected to be complex. Our study demonstrates an unexpected role of DAP10 in the immunoregulatory mechanisms that control the response against the “abnormal self” associated with neoplastic transformation, and suggests that one physiological role of DAP10 costimulation is to activate Tregs to maintain tolerance to self-Ags and, as a result, dampen antitumor immune responses.

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Disclosures
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References


