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*J Immunol* published online 10 August 2011
http://www.jimmunol.org/content/early/2011/08/10/jimmunol.1003879

Supplementary Material  http://www.jimmunol.org/content/suppl/2011/08/10/jimmunol.1003879_9.DC1

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TRAIL/DR5 Plays a Critical Role in NK Cell-Mediated Negative Regulation of Dendritic Cell Cross-Priming of T Cells

Mitsuhiro Iyori,1 Tong Zhang,1 Haddon Pantel, Bethany A. Gagne, and Charles L. Sentman

Dendritic cells (DCs) are critical in initiating immune responses by cross-priming of tumor Ags to T cells. Previous results showed that NK cells inhibited DC-mediated cross-presentation of tumor Ags both in vivo and in vitro. In this study, enhanced Ag presentation was observed in draining lymph nodes in TRAIL−/− and DR5−/− mice compared with that of wild-type mice. NK cells inhibit DC cross-priming of tumor Ags in vitro, but not direct presentation of endogenous Ags. NK cells lacking TRAIL, but not perforin, were not able to inhibit DC cross-priming of tumor Ags. DCs that lack expression of TRAIL receptor DR5 were less susceptible to NK cell-mediated inhibition of cross-priming, and cross-linking of DR5 receptor led to reduced generation of MHC class I–Ag peptide complexes, followed by attenuated cross-priming of CD8+ T cells. In addition, key molecules involved in the TRAIL/DR5 pathway during DC/NK cell interactions were determined. In summary, these data indicate a novel alternative pathway for DC/NK cell interactions in antitumor immunity and may reflect homeostasis of both DCs and NK cells for regulation of CD8+ T cell function in physiological conditions. The Journal of Immunology, 2011, 187: 000–000.

Natural killer cells are an important component of the innate immune system and play critical roles in immune surveillance against tumor cells (1). NK cells recognize tumor cells through both inhibitory and stimulating receptors. Tumor cells that downregulate MHC expression or express high levels of ligands for NK cell-activating receptors become susceptible to NK-mediated lysis. In addition, NK cells can also regulate adaptive immunity, partially through interactions with dendritic cells (DCs) (2, 3).

DCs are the most efficient professional APCs for initiating Ag-specific T cell responses. Cross-priming has been suggested to be a major pathway for tumor Ags to be presented to naive CD8+ T cells because tumor cells usually lack costimulatory molecules as well as adhesion molecules (4). During cross-priming, Ags derived from dead or dying cancer cells are captured by DCs, which process these Ags and present tumor-derived peptides on their MHC class I molecules (4, 5). Tumor Ag peptide-bearing DCs then activate naive CD8+ T cells, a critical step in the development of effective antitumor immunity.

To date, most studies on NK/DC cross-talk are performed in infectious or immunogenic tumor models (often in conjunction with adjuvants). During infection or in the presence of adjuvants with pathogen-associated molecular patterns, DCs and/or NK cells can be activated, promoting a mutually beneficial DC/NK interaction (6–8). Interplay between NK cells and DC have been shown both in vitro and in vivo. Upon stimulation, DCs produce cytokines such as IFN-αβ, IL-12, IL-15, and IL-18, which stimulate NK cells (9–11). Activated NK cells release cytokines, such as IFN-γ, which promote Ag-priming activity by DCs (9). Under certain circumstances, NK cells have been shown to kill immature DC through TRAIL (12). In mice, trimerized TRAIL binds to TRAIL receptor (DR5/TRAIL receptor 2), which results in apoptosis of transformed cells (13). DR5 has also been shown to negatively regulate innate immune responses in DCs (14). Although TRAIL and its receptors have been shown to play important roles in immunosuppressive, immunoregulatory, and immune-effector functions (15), little is known about the interaction, whereas DC and NK cells interplay in less immunogenic tumor models. Our previous results have shown that NK cells inhibited DC activation of Ag-specific CD8+ T cells both in vitro and in vivo using less immunogenic tumor cells (16). In this study, we examined regulatory mechanisms of TRAIL/DR5 cross-linking when NK cells reduced cross-priming by DCs in vitro and in vivo. Our results reveal a novel alternative pathway for DC/NK cell interactions in antitumor immunity, and this pathway may reflect a means for regulation of CD8+ T cell activation in the absence of acute inflammation. This mechanism may be used by tumor cells to limit the induction of immune T cells against tumor Ags.

Materials and Methods

Mice and cell lines

C57BL/6 (B6, wild type [wt]) were purchased from National Cancer Institute (Frederick, MD). Perforin knockout mice C57BL/6-Pf1−/− (Pfp−/−) were obtained from The Jackson Laboratory (Bar Harbor, ME). TRAIL knockout mice (TRAIL−/−) were obtained from J. Peschon (Amgen, Seattle, WA). TRAIL receptor DR5 knockout mice (DR5−/−) were obtained from T. Mak (University Health Network, Toronto, Canada). Male Ag HY-TCR transgenic mice (TCR HY) and chicken OVA-specific, MHC class II-
pressed TCR transgenic OT-II mice were obtained from R. Noelle (Dartmouth Medical School). OVA-specific H-2K<sup>k</sup>-restricted TCR transgenic OT-I mice were obtained from The Jackson Laboratory. B<sub>6</sub>Rag1<sup>–/–</sup> mice were bred and maintained at Dartmouth Medical School. Animals used in experiments were between 7 and 12 wk of age. All experiments were conducted according to protocols approved by Dartmouth College's Institutional Animal Care and Use Committee. Mouse T cell line lymphoma RMA/OVA (H<sup>2</sup>-b), melanoma B16F10/OVA (H<sup>2</sup>-b), and mastocytoma P815/OVA (H<sup>2</sup>-b) cells that express a nonsecreted truncated form of OVA were used for in vivo and in vitro experiments, respectively. H-2K<sup>k</sup>-restricted B<sub>10</sub>3 T cell hybridoma cells that recognize the OVA<sub>257</sub>-264 epitope were obtained from N. Shastri (University of California at Berkeley). Upon activation, B<sub>10</sub>3 cells express the LacZ gene. All cells were grown in RPMI 1640 medium supplemented with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM pyruvate, 10 mM HEPES, 0.1 mM nonessential amino acids, and 50 µM 2-ME.

**Tumor inoculation**

RMA/OVA cells (1<sup>0</sup> or B16F10/OVA cells (5 × 10<sup>5</sup>) were injected s.c. into right flank of mice (day 0). For depletion of NK cells, mice were injected i.p. with 50 µg anti-NK1.1 Ab PK136 (days −2, +3). Control mice were given mouse γ-globulin (Jackson ImmunoResearch, West Grove, PA).

**Cell preparation**

Enrichment of DCs from draining lymph nodes (DLNs), preparation of bone marrow (BM)-derived DCs, H-2K<sup>k</sup>-restricted OVA-specific OT-I T cells, and H-Y T cells were performed, as described (16). Purified NK cells were obtained from B<sub>6</sub>Rag1<sup>–/–</sup> spleen cells using magnetic cell-sorting kit (Miltenyi Biotec, Auburn, CA) and FITC-labeled anti-DR5 mAbs, according to the manufacturer's instructions. NK cell purity was >90%. OVA-reactive OT-II T cells (H<sup>2</sup><sup>−</sup> restricted) were purified in a similar way, except that anti-CD4 mAbs were used. To prepare splenic DCs, spleens were digested with DNase (100 µg/ml; Sigma-Aldrich) at 37°C for 45 min, and light-activated NK cells were depleted with anti-NK1.1 mAbs (clone PK136) and anti-αβ conjugated with biotin (BD Biosciences, San Diego, CA). NK cell purity was >90%. DakoCytomation (Glostrup, Denmark) anti–MHC Class II conjugated peroxidase antibody was used to detect MHC Class II expression on NK cells and DR5 expression on DCs.

**Ex vivo Ag presentation assay**

Nycodenz-enriched lymph node (LN) cells were cocultured with B<sub>10</sub>3 cells (10<sup>5</sup>) at a ratio of 1:5 (LN cells:B<sub>10</sub>3) in 96-well, round-bottom plates for 24 h and stained for LacZ<sup>+</sup> cells, as described previously (17).

**Cross-linking of DR5 with plate-bound anti-DR5 mAb**

Functional grade of anti-DR5 mAbs (MD5-1, 10 µg/ml; eBioscience, San Diego, CA) or control hamster mAbs in PBS was coated on nontissue culture-treated 96- or 24-well plates. The plates were washed twice with PBS, and then Cd11<sup>c</sup><sup>−</sup> BM-DCs in complete media were cross-linked for 4 or 24 h.

**In vitro Ag presentation assay**

Purified CFSE-labeled OT-I or OT-II T cells (5 × 10<sup>5</sup>) were cultured with either 2 × 10<sup>3</sup> BM-DCs or splenic DCs and 2 × 10<sup>4</sup> irradiated P815/OVA (120 Gy) in a total of 200 µl complete medium. In some cases, DCs were pulsed with OVA<sub>257</sub>-264 peptide (10<sup>−10</sup>–10<sup>−12</sup> M) for 45 min at 37°C in complete medium and then washed three times before culture with OT-I or OT-II T cells. In some wells, 2–5 × 10<sup>5</sup> NK cells were added to similar cultures. To stimulate H-Y–specific T cells, DCs from B6 male mice were used. Proliferation of T cells (loss of CFSE labeling in T cells) was determined by flow cytometry after 65–96 h of culture.

**Phagocytosis assay**

BM-DCs were cross-linked with control mAbs or anti-DR5 mAbs for 24 h, and then PKH26-labeled irradiated P815/OVA cells were added to the wells. After 30-min to 24-h incubation, cells were washed with quenching buffer (2 mM EDTA/PBS), stained with allophycocyanin-conjugated anti-Cd11<sup>c</sup> mAbs on ice, and then analyzed by flow cytometry.

**Real-time RT-PCR**

BM-DCs were cross-linked for 4–24 h with control mAb- or anti-DR5 mAb-coated wells or in control wells (PBS). Total RNA was isolated from the cells by using the RNAeasy kit (Qiagen, Valencia, CA). cDNA was synthesized by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and the following primers: GAPDH (forward, 5′-CAGCTGGAAGAACCAAGG-3′; reverse, 5′-GCATTTCCGACCGACAGAAT-3′), reverse, 5′-ATTTCACAGGGGAAATAAGC-3′, inducible NO synthase (nNOS, forward, 5′-GGCAAGCTGTTAGACCTTT-3′; reverse, 5′-TGCATTGGATTGAGGTATTT-3′), arginase-1 (forward, 5′-TGAACACGCGGACAGTTA-3′; reverse, 5′-CGATCCTTGCTTATGTTAC-3′), iNOS (forward, 5′-GATCCTGGAAGAACCAAGG-3′; reverse, 5′-GCTTTCCAAGCCACAG-3′), and galecitin-1 (forward, 5′-TCAACACCTGGGAATGTCTC-3′; reverse, 5′-GGCGAAGATTGAGTGACC-3′). Amplification of GAPDH was done in each experiment. Each cycle threshold (Ct) value of the samples was standardized by GAPDH Ct value (ΔCt), and each ΔΔCt value was normalized to that of 4-h-incubated DCs Ct value (ΔΔCt). Results were shown as relative expression (2<sup>−ΔΔCt</sup>).
treated TRAIL−/− mice (Fig. 1C, 1D). The negative regulation of cross-presentation by NK cells was also observed in a different tumor model by using B16F10/tOVA melanoma cells (Fig. 1E–H). Anti-NK1.1 or control mouse γ-globulin was injected into mice i.p. at days −2 and +3 relative to tumor inoculation at day 0 (C–H). Five days after the inoculation, DC-enriched LNs were cultured with B3Z cells for 24 h. Results are expressed as percentage of LacZ+ B3Z cells per 2 × 10^4 CD11c+ cells and total APC activity per LN. Cumulative data from three independent experiments are shown as mean ± SEM. *p < 0.05, #p < 0.05, compared with DR5−/− mouse IgG, but p > 0.05, compared with B6 mouse IgG.

**FIGURE 1.** DC cross-presentation of tumor Ags was inhibited by NK cells through TRAIL/DR5 in vivo. B6 mice, TRAIL−/− mice, or DR5−/− mice were inoculated with 10^6 RMA/tOVA cells (A–D) or 5 × 10^5 B16F10/tOVA cells (E–H). Anti-NK1.1 or control mouse γ-globulin was injected into mice i.p. at days −2 and +3 relative to tumor inoculation at day 0 (C–H). Anti-NK1.1 or control mouse γ-globulin was injected into mice i.p. at days −2 and +3 relative to tumor inoculation at day 0 (C–H). Five days after the inoculation, DC-enriched LNs were cultured with B3Z cells for 24 h. Results are expressed as percentage of LacZ+ B3Z cells per 2 × 10^4 CD11c+ cells and total APC activity per LN. Cumulative data from three independent experiments are shown as mean ± SEM. *p < 0.05, #p < 0.05, compared with DR5−/− mouse IgG, but p > 0.05, compared with B6 mouse IgG.

Next, experiments were designed to determine whether DR5, a TRAIL receptor, was engaged in DC cross-priming; DLN DCs from DR5−/− mice were compared with DCs from wt B6 mice in the capability of cross-presentation of tumor-associated Ags. It was found that DR5−/− mice-derived DLNs exhibited 2-fold higher Ag presentation on both a per cell basis and total activity in the DLNs compared with those from wt B6 mice (Fig. 1G, 1H). Similar to TRAIL−/− mice, NK cell depletion in DR5−/− mice did not increase CD8+ T cell priming compared with control mAb-treated TRAIL−/− mice (Fig. 1G, 1H). Collectively, these results indicate that NK cells delay the development of specific CTLs in RMA lymphoma and B16F10 melanoma.

**NK cells inhibit DC cross-priming through TRAIL/DR5 in vitro**

NK–DC interactions are generally thought to result in mutual cell activation (2, 3). However, previous data have shown that tumor Ag cross-presentation by DCs was reduced in the presence of NK cells in an in vitro coculture system (16). When OVA-specific H-2Kb-restricted OT-I T cells (CFSE labeled) were cocultured with splenic DCs plus irradiated OVA-expressing P815 cells (P815/tOVA, H-2d) in the presence of NK cells, splenic DC-mediated cross-priming was reduced by NK cells in a dose-dependent manner (Fig. 2A, 2B). Although these tumor cells express OVA, they cannot activate OT-I T cells because they do not express the appropriate MHC class I molecules. To determine which mechanisms NK cells use to inhibit DC cross-presentation function, we tested NK cells purified from TRAIL−/− and perforin−/− mice. It has been reported that TRAIL and perforin are involved in NK cell-mediated killing of immature DCs (12). As shown in Fig. 2C, perforin−/− NK cells showed a similar ability as B6 NK cells to inhibit cross-priming by BM-DCs in a dose-dependent manner, indicating that perforin was not
required for inhibition of DC cross-priming. In contrast, TRAIL \(^{-/-}\) NK cells had a reduced ability to inhibit DC cross-priming (Fig. 2C), suggesting that TRAIL was involved in inhibiting DC-mediated cross-priming. Similar results were observed using splenic DCs (Fig. 2D), but more NK cells were needed to achieve significant inhibitory effects on splenic DC-mediated cross-priming. This difference may be due to the distinct intrinsic features between BM-DCs and splenic DCs. TRAIL \(^{-/-}\) NK cells have been shown to exert both perforin- and FasL-dependent cytotoxicity similar to wild-type NK cells (19, 20). Thus, cross-priming by DCs is most likely regulated by TRAIL itself rather than through an indirect effect of TRAIL deficiency on NK cell development or function. To confirm the vital role of TRAIL in inhibiting DC cross-priming, we generated P815/tOVA cells expressing TRAIL (P815/tOVA-TRAIL). In comparison with P815/tOVA cells, the use of P815/tOVA-TRAIL cells significantly reduced OT-I proliferation in the presence of NK cells (Fig. 2D), suggesting that TRAIL/DR5 interactions play a key role in negative regulation of DC cross-priming.

**NK cells inhibit DC cross-priming of CD4\(^{+}\) T cells with cell-associated Ags, but not soluble Ags**

To determine whether NK inhibition of DC function is restricted to cross-priming, we examined the capability of the DCs to present cell-associated OVA as well as soluble OVA protein by the MHC class II pathway to CD4\(^{+}\) OT-II cells. Exogenous Ags need to be endocytosed and processed before presenting to MHC class II-restricted CD4\(^{+}\) T cells. DC-mediated MHC class II priming of CD4\(^{+}\) T cells to tumor cell-associated Ags was also inhibited by NK cells, although to a lesser extent than presentation to CD8\(^{+}\) T cells (Fig. 3A). Only marginal, but consistent, inhibition (<15%) was observed in DC presentation of soluble OVA proteins to CD4\(^{+}\) T cells (Fig. 3B). Thus, NK cells inhibited the presentation by DCs of cell-associated Ags, but not soluble Ags to T cells.

**NK cells do not inhibit direct priming by DCs**

To test the effect of NK cells on endogenous Ag presentation on MHC class I, T cells purified from H-Y TCR transgenic mice, which are specific for the male minor histocompatibility Ag H-Y, were cocultured with male DCs in the presence of female NK cells. Robust proliferation (~100%) of H-Y transgenic T cells was observed, and NK cells did not inhibit the T cell proliferation (Fig. 4A, 4B). To confirm this, DCs were pulsed with OVA peptide (10\(^{-12}\)–10\(^{-10}\) M) before coculture with OT-I T cells. OVA

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*TRAIL expression on NK cells ( gated on NK1.1) was determined by flow cytometry after coculture with DC and/or tumor cells for 24–72 h. The percentage of TRAIL\(^{+}\) NK cells is shown. The values represent means of two independent experiments.*
peptide-pulsed DCs can directly present peptide to OT-I cells without uptake or processing of Ags. NK cells did not inhibit peptide-pulsed DC-induced T cell proliferation (Fig. 4C) or direct stimulation of OT-I T cells by peptide or anti-CD3 mAbs, whether in the presence or absence of P815 tumor cells (Supplemental Fig. 1).

**Primary nonactivated NK cells do not induce cell death of DCs**

There are several possibilities that may account for NK cell inhibition of DC cross-priming. First, NK cells may kill DCs, which can happen when NK cells are preactivated (e.g., with IL-2) and cultured at higher NK:DC ratios (21). Second, NK cells may affect DC uptake of Ags. To address these possibilities, we first identified the stage(s) at which NK cells affect DC cross-priming. In our in vitro experiments, freshly isolated, nonactivated NK cells were used. These fresh NK cells did not kill P815/tOV A tumor cells (Fig. 5A). To examine whether nonactivated NK cells induced death of DCs, NK cells (10^5) were cocultured with DCs (5×10^4) for 24 and 48 h before being subjected to PI and annexin V staining. Compared with DCs alone, there was no significant (p > 0.05) increase in either apoptosis (annexin V staining positive, PI staining negative) or necrosis (annexin V staining positive, PI staining positive) of DCs (CD11c+) cells after 24 h of coculture with NK cells (Fig. 5B). Interestingly, 48 h after NK and DC coculture, the percentages of both apoptotic and necrotic DCs were reduced when NK cells were present in these cultures compared with DCs alone (Fig. 5B), suggesting that NK cells may provide some survival factors to DCs. These data indicate that NK cells were not killing DCs, but they were most likely affecting Ag uptake and/or Ag processing.

**FIGURE 3.** NK cells inhibit DC-mediated MHC class II-restricted priming of CD4+ T cells with tumor Ags, but not soluble Ags. CFSE-labeled OVA-specific OT-II (CD4+, I-Ab-restricted) T cells were cocultured with splenic DCs and gamma-ray–irradiated tumor cells (P815/t-OVA) (A) or soluble OVA protein (100 μg/ml) (B). After 2.5 d, proliferation of CD4+ T cells was determined by flow cytometry. Numbers represent normalized percentage of proliferating T cells (no NK = 100%). Cumulative data from three independent experiments are shown as mean ± SEM. *p < 0.05.

**FIGURE 4.** NK cells do not inhibit DC direct priming. A and B, CFSE-labeled H-Y–specific T cells were cocultured with male splenic DCs for 2.5 d in the presence of NK cells (2–50×10^3). Percentage (A) or MFI (B) of proliferating T cells (gated on CD8b+ cells) is shown as mean ± SEM of triplicates. C, OVA peptide (SIINFEKL, 10^{-10}–10^{-12} M)-pulsed BM-DCs (2×10^5) were cocultured with CFSE-labeled OT-I T cells (5×10^4) for 2.5 d in the presence or absence of NK cells (2–50×10^3/well). Numbers represent percentage of proliferating T cells. A representative result of two independent experiments is shown as mean ± SEM of triplicates. D, CD11c+ BM-DCs (5×10^5) were cultured for 24 h in control mAbs or anti-DR5 mAb-coated 24-well plates or in control wells (PBS). The cells (2×10^5) were pulsed with OVA peptide (10^{-10}–10^{-12} M), and then cocultured with CFSE-labeled OT-I T cells. Proliferation of CD8b+ T cells was determined by flow cytometry. Cumulative data from three independent experiments are shown as mean ± SEM.

**FIGURE 5.** Resting NK cells do not kill tumor cells or DCs. A. Splenocytes from RAG-1−/− mice were incubated with or without IL-4 (1000 U) for 4 d, and then cocultured with P815/OVA cells or YAC-1 cells at ratios from 1:1 to 25:1 in 5-h 51Cr release assays. Cumulative data from three independent experiments are shown as mean ± SEM. B. Primary nonactivated NK cells do not induce cell death of DCs. DCs (BM-DCs, 5×10^5) alone or NK (10^5) plus DCs were cultured in 96-well plates for either 24 or 48 h before annexin V staining. The data shown are gated on DCs (allophycocyanin anti-CD11c+). As positive control, 5% EtOH was added 24 h before cell staining. A representative result of two independent experiments is shown as mean ± SEM of triplicates.
DR5 inhibits DC cross-priming in vitro by reducing phagocytic activity of tumor cells

To get insight into the molecular mechanisms of DR5-mediated negative regulation of cross-priming, BM-DCs were cultured for 24 h in anti-DR5 mAb (MD5-1)-coated wells (anti-DR5-treated DC), control mAb-coated wells (control Ab-treated DC), or non-Ab-coated wells (PBS-treated DC). It was found that control Ab-treated DCs as well as PBS-treated DCs effectively cross-primed OT-I T cells in the presence of P815/OVA cells, but anti-DR5–treated DCs significantly attenuated the cross-presentation (Fig. 6A), which is consistent with the inhibitory effects of NK cells (Fig. 2). To examine whether this inhibitory effect was restricted to the cross-priming of tumor cell-associated Ags, the DCs were pulsed with OVA peptide and cocultured with OT-I T cells. The results showed that DR5 did not affect the capability of Ag presentation by OVA peptide-pulsed DCs (Fig. 4D), indicating that DR5 attenuated DC cross-presentation of cellular Ags, but not DC direct peptide presentation.

As the first step in cross-priming of tumor cell-associated Ags, DCs need to phagocytose tumor cells. To analyze this step, BM-DCs were cocultured with PKH26+ P815/OVA cells for different periods of time and then analyzed by flow cytometry. The CD11c+PKH26+ population represents the DCs that phagocytosed tumor cells (Fig. 6B). Both percentage of phagocytosis and mean fluorescent intensity (MFI) of PKH26 were enhanced in a time-dependent manner (Fig. 6B, 6C). It was found that percentages of phagocytosis-positive cells showed no significant difference between control Ab-treated DCs and anti-DR5–treated DCs (Fig. 6D). Intriguingly, the uptake of P815/OVA by anti-DR5–treated DCs was attenuated in earlier time points (30 min and 4 h), compared with that of control Ab-treated DCs (Fig. 6E), suggesting that phagocytic activity of individual DCs for tumor cells was delayed by DR5 cross-linking.

Because the TRAIL/DR5 pathway showed negative regulation of DC cross-priming both in vivo and in vitro, the activation of DR5 on the generation of MHC–Ag peptide complexes on the cell surface of DCs was determined. To this end, BM-DCs were cultured in the anti-DR5 mAb-coated wells in the presence of irradiated P815/OVA cells for 24 h. Cross-linking of DR5 with anti-DR5 mAbs resulted in lower amounts of K\(^{\text{b}}\)-OVA complexes compared with control mAb-treated DCs (Fig. 6F). In addition, treatment anti-DR5 mAbs did not affect the ability of DR5\(^{-/-}\) DCs to present K\(^{\text{b}}\)-OVA complexes on cell surfaces (Fig. 6G), indicating that DR5 reduced cross-presentation activity of tumor cell-associated Ags.

**FIGURE 6.** DC cross-priming of tumor cells is inhibited by cross-linking with anti-DR5 mAbs. A, CD11c+ BM-DCs (5 \times 10^5) were cultured for 24 h in control mAbs or anti-DR5 mAb-coated 24-well plates or in control wells (PBS). The DCs (2 \times 10^5) were then cocultured for 4 d with CFSE-labeled OVA-specific OT-I T cells (5 \times 10^5) and P815/OVA cells (2 \times 10^5). Numbers represent percentage of proliferating T cells. A representative result of four independent experiments is shown as mean \pm SEM of triplicate. B, BM-DCs (5 \times 10^5) were cultured for 24 h in control mAbs or anti-DR5 mAb-coated 96-well plates and then incubated with P815/OVA cells (5 \times 10^5, PKH26+). Percentages refer to PKH26+ cells within the CD11c+ gated cells (DCs). The MFI of PKH26 (C, E) and percentages of PKH26-positive DCs (D) are indicated for each time point (30 min, 1 h, and 4 h). Cumulative data from three independent experiments are shown as mean \pm SEM. The wt (F) or DR5\(^{-/-}\) (G) BM-DCs alone or DCs with tumor cells were cocultured for 24 h before staining with 25-D1.16 and anti-CD11c mAbs. A representative result of two independent experiments is shown as mean \pm SEM of triplicates. *p < 0.05, ***p < 0.001.
Arginase-1 is involved in DR5-mediated inhibition of DC cross-priming

The tumor microenvironment is abundant in molecules suppressing differentiation and function of both DCs and T cells, including vascular endothelial growth factor, IL-6, IL-10, TGF-β, M-CSF, arginase, IDO, PGE2, cyclooxygenase-2, and NO synthase (25, 26). Because interaction of TRAIL and TRAIL receptors has been shown to regulate production of cytokines and chemokines in an NF-κB–dependent manner (27, 28), it is possible that the suppressive cytokines and chemokines were induced by cross-linking of anti-DR5 mAbs and involved in the inhibitory effects of DC cross-priming as secondary factors. As shown in Fig. 8A, mRNA expression of IL-10, iNOS, and arginase-1 in BM-DCs was upregulated in response to cross-linking of anti-DR5 mAbs. The upregulation of IL-10 in DCs was induced by DR5 at an early time point (4 h), whereas iNOS and arginase-1 were induced after 24 h. Cross-linking with anti-DR5 mAbs on DCs did not affect the mRNA expression of IDO1 and galectin-1 (Fig. 8A). To assess whether the inhibitory molecules were involved in the DR5-mediated inhibition of DC cross-priming, experiments were carried out to determine whether inhibitors specific for these molecules affected DC cross-priming. The results showed that the iNOS inhibitor L-NIL significantly enhanced both control Ab-treated DC and anti-DR5 mAb cross-priming (Fig. 8B). In contrast, the arginase inhibitor nor-NOHA did not affect control Ab-treated DC cross-priming, but significantly increased anti-DR5 mAb cross-priming. The IDO inhibitor 1-MT and the anti–IL-10 blocking Abs had no effect on the DC cross-priming (Fig. 8B). Taken together, these data suggest that DR5-mediated induction of arginase-1 negatively regulates DC cross-priming of tumor-associated Ags to CD8+ T cells.

Discussion

The data presented in this study demonstrate that freshly isolated NK cells inhibit DC cross-priming, but not direct priming in a TRAIL/DR5-dependent manner. NK cell-derived TRAIL, but not perforin, was the key mechanism for inhibition of DC-mediated cross-priming both in vitro and in vivo. Consistent with these data, the TRAIL receptor DR5-deficient DCs were less susceptible to NK-mediated inhibition on DC cross-priming. In addition, cross-linking with anti-DR5 mAbs attenuated the capability of DC cross-priming by reducing phagocytosis and through the induction of arginase.

NK cells have been shown to be important not only in elimination of tumors and virus-infected cells, but also in modulation of adaptive immunity. One of the mechanisms in which NK cells regulate adaptive immunity is through cross-talk with DCs. DCs can activate NK cells via production of IL-12, IL-15, and type I...
activated DCs, such as CD40L and IFN-γ production by activated DCs and cell–cell contacts promote effective maturation of DCs (7, 21). In some cases, NK cells may limit DC activity by killing immature DCs through either TRAIL-mediated or Nkp30-mediated mechanism (12, 31). Another possible regulatory role played by NK cells is mediated through the interaction between the class I b MHC molecule Qa-1-Qdm on activated T cells and CD94/NKG2A inhibitory receptors expressed by NK cells in some autoimmune disease models, such as experimental autoimmune encephalomyelitis (32).

Activated, but not resting, human NK cells have been shown to be capable of killing immature autologous DCs at a high NK/DC ratio (21). Hayakawa et al. (12) showed that freshly purified hepatic NK cells mediated lysis of immature DCs in vitro in a TRAIL-dependent manner, although data suggest that perforin may be partially involved in the elimination. In their study, hepatic NK cells were used that constitutively express TRAIL (12, 20). In this study, purified splenic NK cells were used, and these resting splenic NK cells do not express TRAIL. After coculture with DCs, TRAIL expression was induced (Table I). In addition, using OVA peptide-pulsed DCs, we demonstrate that as long as OVA peptide–TRAIL expression was induced (Table I). In addition, using OVA splenic NK cells do not express TRAIL. After coculture with DCs, may be partially involved in the elimination. In their study, hepatic NK cells were used that constitutively express TRAIL (12, 20). In this study, purified splenic NK cells were used, and these resting splenic NK cells do not express TRAIL. After coculture with DCs, TRAIL expression was induced (Table I). In addition, using OVA peptide-pulsed DCs, we demonstrate that as long as OVA peptide–MHC complexes were expressed on DCs, NK cells did not inhibit DC-induced T cell proliferation, indicating that freshly isolated, nonactivated NK cells do not kill DCs. Moreover, in two tumor models in vivo, we demonstrate that NK cells inhibited cross-priming of tumor Ags in the DLN.

Multiple factors can affect DC cross-priming. Molecules that stimulate DCs, such as CD40L and IFN-αβ, have been shown to enhance cross-priming (5, 33). The immunogenic properties of Ags might also affect DC cross-priming of T cells (34, 35). In addition, another study suggests that activated tumor-infiltrating NK cells are crucial for cross-priming of B16 melanoma cells by TLR9-activated plasmacytoid DCs (36). In our experiments, less immunogenic RMA, B16F10, and P815 cells were used, and these tumor cells are rather resistant to primary resting NK cells. The lack of TLR signals may account for the discrepancy of results using different models. TLR activation may cause regression of tumors by increasing vascular permeability and by recruiting leukocytes, resulting in tumor lysis by infiltrating NK and cytotoxic T cells (37). Furthermore, DC/NK contact is essential for the induction of polyIFN-mediated antitumor NK cells (38). However under physiological conditions, the function of resting NK cells in antitumor immunity remains to be elucidated. Present study indicates the unique homeostasis that resting NK cells negatively regulate establishment of tumor immunity by immature DCs.

There are at least two distinct pathways by which the DC cross-priming occurs, as follows: the phagosome-to-cytosol pathway and the vacuolar pathway (Fig. 9) (5). It is possible that NK cells may affect DC cross-priming during steps from Ag transfer from the phagosome to cytosol, Ag degradation by the proteasome, peptide transport by TAP, and peptide loading in the endoplasmic reticulum (Fig. 9). Presentation of native OVA-coupled beads is TAP dependent and implicated in the phagosome-to-cytosol pathway (39), whereas soluble Ag is found to be cross-presented in the vacuolar pathway (40), and both pathways contributed to the presentation of some Ags such as polyactic-coglycolic acid-OVA (41, 42). DR5 did not inhibit cross-priming of soluble OVA as well as OVA-coupled beads in this study, indicating that DR5-mediated inhibition of cross-priming is restricted to tumor cell-associated Ags. We showed that DR5−/− DCs are resistant to NK cell inhibition of DC cross-priming in comparison with wt B6 DCs, and that DR5 cross-linking reduced generation of Kb-OVA complex on DCs, further supporting the critical role of TRAIL/DR5 regulating DC cross-priming of tumor Ags. T cells may express DR5, which could lead to blockade of T cell cycle progression or T cell death (43, 44). In this study, T cell proliferation was used as readout for DC cross-priming, but NK cells did not inhibit peptide-pulsed DC-induced or anti-CD3 mAb-induced direct T cell proliferation indicating that NK cells did not directly affect T cell proliferation, as previously reported (16).

DR5 can function as a negative regulator of DCs rather than inducing apoptosis of DCs (14). DR5−/− DCs produced more IL-12 and TNF-α upon stimulation by TLR agonists. The role of DR5 in TLR signaling is most likely due to the regulation of IkB-α degradation or stability, leading to the induction of immunoregulatory molecules. In this study, enhanced expression of IL-10, iNOS, and arginase-1 in DCs by DR5 cross-linking indicates that DR5 pathway can create immunosuppressive circumstances that protect the tumors from immune attack. The imbalances of immunological reactions in the tumor microenvironment may result in immune tolerization (25). To date, roles of arginase-1 in DC cross-priming have not been well defined, although this molecule has been demonstrated to inhibit T cell effector functions (45). Our data showed that the reduced cross-priming by DR5 cross-linking was specifically reversed by the arginase-1 inhibitor NOHA. Thus, these data support the concept that activation of arginase-1 downstream of DR5 leads to suppression of DC cross-priming for tumor cells.

Tumor cells are “altered self” (46). The immune system can efficiently recognize immunogenic tumor cells when a proinflammatory environment is induced (2, 47). However, some less immunogenic tumor cells can evade immunosurveillance via weak self-Ag expression or local immune suppressive mechanisms (46). Without proinflammatory signals, a NK/DC positive feedback loop may not be efficiently initiated, so that tumor-induced tolerance may prevail (8, 48). The detailed mechanisms for DC cross-tolerance induction remain unclear, and DC-derived signals are most likely a key step in the initiation of immunity or maintenance of tolerance. Under normal circumstances, tissues are being remodeled, cell death occurs, and macrophages and other leukocytes repair and help maintain tissue architecture and function. DCs are constantly bringing Ags into LNs derived from self-
Ags, which should not induce an immune response in the absence of an inflammatory trigger. The data presented in this study suggest that DCs may use NK cells to help regulate this process. Tumor cells can take advantage of this NK cell-mediated inhibition of DC cross-priming and use it to prevent or delay the induction of adaptive immunity. Thus, the data may reflect a normal immune homeostatic mechanism that regulates the initiation of adaptive immunity within LN. Our results support the hypothesis that NK cells negatively regulate DC-mediated cross-priming through TRAIL/DR5. These data provide new insights into NK-mediated regulation of adaptive immunity.

Acknowledgments

We thank Drs. Jacques Peschon (Amgen, Seattle, WA) and Tak Mak (University Health Network, Toronto, Canada) for providing TRAIL−/−and DR5−/−mice, respectively; Dr. Randolph J. Noelle for providing OT-II and H-Y transgenic mice; Alice Givan and Gary Ward for assistance with flow cytometry; and the staff of the Animal Resources Center for assistance with animal care.

Disclosures

The authors have no financial conflicts of interest.

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