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Adiponectin Deficiency Suppresses Lymphoma Growth in Mice by Modulating NK Cells, CD8 T Cells, and Myeloid-Derived Suppressor Cells

Sora Han,* Ae Lee Jeong,* Sunyi Lee,* Jeong Su Park,* Kwang Dong Kim,† Inpyo Choi,‡ Suk Ran Yoon,‡ Myung Sok Lee,* Jong-Seok Lim,* Seung Hyun Han,‡ Do Young Yoon,* and Young Yang*

Previously, we found that adiponectin (APN) suppresses IL-2–induced NK cell activation by downregulating the expression of the IFN-γ–inducible TNF-related apoptosis-inducing ligand and Fas ligand. Although the antitumor function of APN has been reported in several types of solid tumors, with few controversial results, no lymphoma studies have been conducted. In this study, we assessed the role of APN in immune cell function, including NK cells, CTLs, and myeloid-derived suppressor cells, in EL4 and B16F10 tumor-bearing APN knockout (KO) mice. We observed attenuated EL4 growth in the APNKO mice. Increased numbers of splenic NK cells and splenic CTLs were identified under naive conditions and EL4-challenged conditions, respectively. In APNKO mice, splenic NK cells showed enhanced cytotoxicity with and without IL-2 stimulation. Additionally, there were decreased levels of myeloid-derived suppressor cell accumulation in the EL4-bearing APNKO mice. Enforced MHC class I expression on B16F10 cells led to attenuated growth of these tumors in APNKO mice. Thus, our results suggest that EL4 regression in APNKO mice is not only due to an enhanced antitumor immune response but also to a high level of MHC class I expression. The Journal of Immunology, 2013, 190: 4877–4886.

Adiponectin (APN) is named for its secretion in adipose depots and its hormone functions. The expression of APN correlates negatively with occurrences of obesity and diabetes. Additionally, APN has anti-inflammatory and antitumor functions. The tumor-associated function was initially studied because of the association between obesity and breast cancer risk. The study results revealed that low serum APN levels were significantly associated with an increased risk for breast cancer and a biologically aggressive tumor phenotype (1). Numerous studies have demonstrated that serum levels of APN are significantly low in various tumors, including endometrial (2), gastric (3), colorectal (4), and prostate (5) cancers, chronic lymphocytic leukemia, and myeloproliferative diseases (6).

In APN knockout (KO) mice on a C57BL/6 background, mouse mammary tumor virus/polyoma virus middle T–induced tumors had delayed onsets and slower growth rates due to the loss of the proangiogenic APN (7), whereas insufficient APN production in mice from FVB/N and C57BL/6J backgrounds promoted mammary tumor onset and progression by downregulating phosphate and tensin homolog activity and activating the PI3K/Akt signaling pathway (8). Additionally, APN-null mice of the FVB/N and C57BL/6J mixed backgrounds had significantly slower tumor growth rates during the early disease stages, but the tumors were more aggressive during the advanced disease stages (9). In contrast, when B16F10 cells or Lewis lung carcinoma cells were s.c. injected into APN-null mice, the growth of both tumors was promoted in response to reduced macrophage recruitment to the tumors; however, this did not affect cancer cell mitosis, apoptosis, or tumor-associated angiogenesis (10). Meanwhile, APNKO mice were protected from dextran sulfate sodium– and trinitrobenzene sulfonic acid–induced colonic inflammation (11). Thus, APN has a proinflammatory role in colitis and anti-inflammatory effects in atherosclerosis (12). However, another group reported that in a chronic inflammation-induced colon cancer model, APNKO mice had higher levels of immune cell infiltration and inflammation than did wild-type (WT) mice (13). Until now, there have been discrepancies regarding the role of APN in inflammation and tumor models. Thus, more studies on the role of APN in tumor initiation, progression, metastasis, and immune cell regulation are needed for a complete understanding of its function.

Several lymphoma and leukemia studies have examined the serum levels of APN. Serum APN levels were increased in adult non-Hodgkin lymphoma (NHL) and childhood NHL patients, and the elevated APN levels were positively associated with poor prognosis (14–16). Meanwhile, serum APN levels were inversely associated with occurrences of acute myeloblastic leukemia, but
not acute lymphoblastic leukemia (17). Although molecular studies of the antitumor function of APN have been reported for several solid tumor types, to our knowledge, few molecular studies have been conducted on the role of APN in lymphoma. In our previous study, we found that APN inhibited the cytotoxicity of NK cells in vitro (18). Hence, we hypothesized that a high serum level of APN in NHL patients might result in a poor prognosis and that lymphoma growth might be attenuated because of enhanced NK cytotoxicity in the APKNO mice. In this study, we assessed the role of APN in tumor growth and in the functions of immune cells, including NK cells, CTLs, and MDSCs, in EL4 lymphoma and B16F10 melanoma tumor-bearing APKNO mice.

Materials and Methods

Reagents and cell culture

RBC lysis buffer, collagenase type II, indomethacin, gentamicin, and G418 were purchased from Sigma-Aldrich. CFSE and mouse stem cell factor were purchased from Invitrogen. Mouse IL-2, IL-4, IL-7, IL-15, Flt3 ligand, GM-CSF, G-CSF, and IFN-γ were purchased from PeproTech. Mouse APN was purchased from PeproTech. The EL4 and YAC-1 cell lines were cultured in RPMI 1640 medium that was supplemented with 10% heat-inactivated FBS, and the B16F10 melanoma cell line cultured in DMEM was supplemented with 10% heat-inactivated FBS. Cultured cells were incubated at 37°C, 5% CO2.

Mice

APKNO mice on the C57BL/6d background were purchased from The Jackson Laboratory. We used 6- to 12-wk-old male APKNO mice and age-matched C57BL/6d male mice as controls. Bedding was changed once a week, and the temperature and humidity were controlled. Mice were housed under 12-h light/dark conditions and allowed free access to food and water. The plans and protocols for the animal experiments were approved by the Institutional Animal Care and Use Committee of Sookmyung Women’s University, Seoul, South Korea.

Abs and flow cytometric analysis

Cells from individual tissues were stained with the following FITC-, PE-, PE-Cy7-, allophycocyanin-, or Alexa Fluor 647-conjugated anti-mouse Abs: anti-CD3ε, anti-NK1.1, anti-Ly49D, anti-NKG2A/C/E, anti–IFN-γ, anti-IgG2a isotype, anti–H-2Kb/H-2Db (BioLegend), anti-Ly49A, anti-NKG2D (BD Pharmingen), anti-CD4, anti-NKG2A/B6, anti-CD8a, anti-CD11b, anti-Ly6G, anti-Ly6C, anti-F4/80, and -IgG2b isotype (eBioscience) in PBS with 1% FBS for 30 min at 4°C. The labeled cells were analyzed on a FACSCanto II cytometer equipped with FACSDiva software (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Cell isolation from spleens, bone marrow, and solid tumors

Spleens, tibias, and femurs were extracted from mice, and single cells were harvested with a 70-μm nylon cell strainer (BD Falcon) after the residual erythrocytes had been lysed with RBC lysis buffer. Solid tumors were incubated with collagenase type II to isolate the cells. The viability and numbers of isolated cells from the individual tissues were determined by trypan blue exclusion counts.

Tumor control in vivo

EL4 or B16F10 cells at a concentration of 2 × 10^7/ml PBS were injected s.c. into mice, and tumor growth was monitored by measuring the tumor volumes (length × width) with calipers. We performed an in vivo tumor control assay as described previously (19), with modifications. Briefly, EL4 or B16F10 cells were labeled with 10 μM CFSE (Invitrogen) at 37°C for 10 min, and the reaction was stopped by the addition of 5 vol culture medium. Cells were washed twice with PBS, and 1 × 10^7 EL4 or B16F10 cells in 400 μl PBS were administered i.p. After 24 h, the peritoneal cells were recovered. EL4 lymphoma and B16F10 melanoma cells were distinguished in a flow cytometric analysis by forward versus side scatter analysis and CFSE labeling.

NK cell and CTL depletion in vivo

In vivo NK cell depletion was performed as described previously (20), with slight modifications. Briefly, mice were injected i.p. with 50 μl rabbit anti–asialo-GM-1 Ab (Wako Chemicals) 1 d before the tumor inoculations and were injected with an additional 100 μl of this Ab after the tumor inoculations. For CTL depletion in vivo, 100 μl 1 mg/ml goat anti-CD8 was injected i.p., and the tumor cells were inoculated 3 d after the anti-CD8 Ab administration. An additional 100 μl 2 mg/ml anti-CD8 was injected 1 d after the tumor inoculations.

NK cell purification by magnetic cell sorting

NK cells were isolated from freshly isolated mouse spleenocytes with an NK cell isolation kit (Miltenyi Biotec), according to the manufacturer’s protocol. To determine cell purity by flow cytometry, we stained the negatively isolated NK cells with FITC-labeled goat anti-mouse NK1.1. NK cell purity exceeded 95%.

NK cytotoxicity assay

NK cell cytotoxicity was measured as described previously (14), with the following modification. NK cells were stimulated with IL-2 (100 U/ml; PeproTech) for 24 h, harvested, and washed twice. Viable NK cells were counted with the trypan blue dye exclusion method, and equal numbers of viable NK cells were used as effectors in a 51Cr-release assay. YAC-1 target cells were incubated with 1.5 μCi 51Cr at 37°C for 1 h, which allowed the 51Cr-labeled YAC-1 cells to be used as target cells, and the NK cells were distributed in triplicate at E:T ratios of 5:1 to 1:1. The cocultures were incubated for 3 h at 37°C in 5% CO2. After 4 h, the supernatants were harvested, and the released radioactivity was counted with a scintillation counter (Ractobeta). The percentage of sp. act. was calculated with the following equation: % specific release = ([experimental release − spontaneous release]/maximum release − spontaneous release) × 100.

Differentiating NK cells from hematopoietic stem cells in vitro

NK differentiation from hematopoietic stem cells (HSCs) was performed as previously described (19, 21). Briefly, c-Kit+, lineage− B cells (B220), T/ NK cells (CD2), granulocytes (Gr-1), monocytes (CD11b), and erythrocyte (TER-119)–depleted HSCs were purified with a magnetic cell sorting and separation kit (Miltenyi Biotec), according to the manufacturer’s protocol. The indicated Abs were purchased from BD Pharmingen. The purified HSCs were seeded onto 24-well plates (Corning Life Science) at 1 × 10^6 cells/well and cultured for 6 d in RPMI 1640 medium that was supplemented with 30 ng/ml mouse stem cell factor, 50 ng/ml mouse Flt3 ligand, 0.5 ng/ml mouse IL-7, 2 μg/ml indomethacin, and 20 μg/ml gentamicin at 37°C, 5% CO2. The culture medium was refreshed every 3 d, and 5 μg/ml rAPN was added to the cultures as needed on day 3. To generate mature NK (mNK) cells, we cultured OP9 stromal cells with 50 ng/ml mouse IL-15. After 4–6 additional days of culture, the numbers of NK1.1+ cells were determined by flow cytometric analysis after staining with anti-CD122 and anti-NK1.1 Abs.

Ex vivo MDSC differentiation

For MDSC differentiation, bone marrow cells were prepared as follows: 3 × 10^6 cells were seeded onto 24-well plates (Corning Life Science) and cultured for 5 d in RPMI 1640 medium that was supplemented with 10 ng/ml mouse GM-CSF and 10 ng/ml mouse IL-4 at 37°C, 5% CO2. After 3 d in culture, the floating cells were removed and the media were refreshed. We added 5 μg/ml rAPN and 100 ng/ml G-CSF to the cultures as needed. After 5 d, the numbers of MDSCs were determined by flow cytometric analysis after staining with anti-CD122 and anti-NK1.1 Abs.

rAPN protein purification

CHO-K1 cells (a gift from Professor Myeong Sok Lee, Sookmyung Women’s University, Seoul, South Korea) were transfected with an APN expression plasmid. A stable cell line was selected with 2 μg/ml G418 (Sigma-Aldrich). The supernatant was collected when the cells became confluent on a 10-cm cell culture plate (Corning Life Science). APN protein was concentrated from the supernatants with Amicon Ultra centrifugal filter units (Millipore). The concentration of the purified APN protein was determined with a bicinechonic acid protein assay kit (Pierce).

Mouse cytokine assay

A mouse cytokine assay was performed on mouse serum from EL4 tumor-bearing mice, and the assay was conducted with the Mouse Cytokine Array Panel A (R&D Systems), according to the manufacturer’s protocol. Denaturation analysis was performed on the blots with Multi Gauge v3.0 software (Fujifilm).
RT-PCR analysis
Total RNA samples were prepared from HSCs and MDSCs with RNAiso Plus (TaKaRa). The prepared total RNAs were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) at 37°C for 1 h. A PCR analysis was performed to amplify the mRNAs that encoded the APN receptor 1 (AdipoR1), APN receptor 2 (AdipoR2), T-cadherin, and inducible NO synthase (iNOS) with the following appropriate primer pairs: AdipoR1 forward, 5’-TTCCTGGCTCTTCCACAT-3’, reverse, 5’-GA-CAAAAGCTTACGGATAG-3’; AdipoR2 forward, 5’-ATTCTCCCAAA-AGAGGGGA-3’, reverse, 5’-TGTTGCAATAATGCTGCTTG-3’; T-cadherin forward, 5’-ACATCTCTGTCGGACCTG-3’, reverse, 5’-AG-TGTTCTGCGATCTACGCA-3’; and iNOS forward, 5’-TCCACTTC-GAGGCGCCGCA-3’, reverse, 5’-TGCGTGCGAAACGGAGGCAG-3’. Amplifications of AdipoR1/R2, T-cadherin, and iNOS transcripts were performed during 30 PCR cycles. Each PCR amplification cycle included a denaturation step at 95°C for 30 s, and an extension step at 72°C for 30 s.

Protein extraction and Western blotting
Protein samples were prepared with ice-cold RIPA buffer that contained 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% NaDodSO4, and a protease inhibitor mixture (Sigma-Aldrich). The total cell lysates were centrifuged at 13,500 rpm for 15 min at 4°C to remove insoluble materials. The protein extracts were resolved by 12% NaDodSO4-PAGE at 130 mA for 1 h, and the gels were transferred to nitrocellulose membranes. The membranes were blocked for 30 min at ambient temperature with 5% skim milk (Invitrogen) in TBS that contained 50 mM Tris-HCl and 150 mM NaCl (pH 7.6) and were subsequently incubated with 1:1000 dilutions of a rabbit anti-mouse APN primary Ab overnight at 4°C and a HRP-conjugated anti-goat secondary Ab for 2 h at ambient temperature. Peroxidase activity was visualized using an ECL kit (Roche Diagnostics).

Statistics
Statistical analyses were performed with a paired Student t test and a one-way factorial ANOVA; the Tukey and Scheffé tests were also used. The maximum level of significance of p < 0.05 was used for all statistical comparisons. Data are presented as means ± SD. The FACS data shown are representative of at least three separate experiments.

Supplemental materials
Supplemental Table I shows the changes in lymphocyte populations in the naïve and EL4 tumor-bearing APNKO mice. Supplemental Fig. 1 shows the effects of APN on antitumor activity in female and obese mice. Supplemental Fig. 2 shows the in vitro effects of APN on B16F10 and EL4 cell proliferation and on T-cadherin mRNA expression in immune cells in vivo. Supplemental Fig. 3 shows the increased frequencies of Ly49D-expressing CTLs in tumor-bearing APNKO mice.

Results
EL4 lymphoma growth was suppressed in APNKO mice
Previously, we determined that APN could inhibit NK cell cytotoxicity. Hence, we hypothesized that tumor growth would be attenuated by enhanced NK cytotoxicity in APNKO mice. To test this hypothesis, we s.c. administered EL4 lymphoma cells to APNKO mice, which do not produce APN (Fig. 1A), and measured the tumor growth. As expected, EL4 tumor growth was attenuated in the APNKO mice. On day 21, the average tumor weight in APNKO mice was ~50% less than in WT mice (Fig. 1B). To determine whether the serum level of APN correlated proportionately with EL4 tumor growth, we compared the growth rates of lymphomas between male and female mice because female mice are known to have higher serum levels of APN. A modest increase in EL4 tumor growth was observed in female mice compared with male mice (Supplemental Fig. 1A). Next, we used APN heterozygote (HZ) mice and obese mice to measure the growth rates of EL4 tumors. Growth rates were increased in WT mice compared with obese mice and in APNHZ mice compared with APNKO mice. These results indicate that EL4 tumor growth is proportionally related to the serum levels of APN in vivo (Supplemental Fig. 1B). Because EL4 tumor growth was inhibited in the absence of APN, it is possible that APN could increase EL4 cell proliferation in vitro (22). To determine whether APN directly affected EL4 cell proliferation, EL4 cells were treated with rAPN. APN treatment did not affect EL4 cell proliferation (Supplemental Fig. 2A). Finally, to exclude the possibility that APN failed to affect EL4 cell proliferation owing to the absence of APN receptor expression, we determined the levels of AdipoR1/R2 and T-cadherin mRNA expression in EL4 cells by RT-PCR. EL4 cells expressed AdipoR1/R2 and modestly expressed T-cadherin (Supplemental Fig. 2B).

Splenic NK cell populations under tumors-free conditions and CTL populations under tumor-challenged conditions are greater in APNKO mice
APN might retard EL4 tumor growth by regulating tumor-killing immune cells, such as NK cells and CTLs, rather than by affecting tumor cells directly through the APN molecular signaling pathway. To determine the role of APN in immune cell regulation, we characterized the immune cell populations in EL4 tumor-bearing APNKO mice. In naïve APNKO mice, the splenic NK cell population was ~29% higher than in WT mice (Fig. 1C) but was significantly reduced at 21 d after EL4 tumor challenge. Meanwhile, no significant differences in the splenic CTL populations were identified between naïve APNKO and WT mice. However, upon EL4 tumor challenge, the CTL population increased by nearly 2-fold in APNKO mice when compared with WT mice (Fig. 1D). No differences in the proportion of splenic regulatory T cells were noted between APNKO and WT mice under either condition (Supplemental Table I). These findings indicate that increased numbers of NK cells in the APNKO mice might contribute to the initial stages of EL4 tumor formation and that increased CTL populations after tumor challenge might inhibit EL4 tumor growth at later stages.

Enhanced NK cell and CD8+ T cell populations are associated with EL4 lymphoma growth regression in APNKO mice
Because the NK cell population was increased in APNKO mice, we compared the cytolytic activities of NK cells from APNKO and WT mice. NK cells were isolated by magnetic cell sorting, treated or not with IL-2 for 24 h, and incubated with Cr51-labeled YAC-1 target cells, after which the released Cr51 concentrations were determined as a measure of cytolytic activity. Interestingly, the IL-2-treated APNKO NK cells had significantly increased cytolytic activity levels, and even the basal activity levels of NK cells from APNKO mice were higher than those from WT mice (Fig. 2A). Next, to determine whether the increased cytolytic activity was responsible for the delayed EL4 tumor growth in APNKO mice, we depleted NK cells in these mice with an anti-asialo-GM1 Ab. The numbers of NK cells were dramatically reduced after the treatment with anti-asialo-GM1 Ab overnight at 4°C and a HRP-conjugated anti-goat secondary Ab for 2 h at ambient temperature. Peroxidase activity was visualized using an ECL kit (Roche Diagnostics).

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depleted APNKO mice and found that the CTL population was reduced by 50% (Fig. 2E). Thus, CTLs might be associated with reduced EL4 tumor growth in APNKO mice. To test this possibility, CD8+ T cells were depleted with an anti-CD8 Ab. The number of CD8+ T cells was dramatically reduced by treatment (Fig. 2F), but the NK cell population was not affected (Fig. 2G). The CD8+ T cell–depleted APNKO mice were injected s.c. with EL4 cells, and the tumor growth rate was greatly enhanced in the CD8+ T cell–depleted APNKO mice when compared with the control APNKO mice (Fig. 2H).

Expression of the NK cell–activating receptor Ly49D is increased in APNKO mice and downregulated by APN

Next, we determined the effects of APN on NK cell– and CD8+ T cell–mediated antitumor killing by examining the inhibitory and activating receptors that were expressed on splenic NK cells from APNKO and WT mice. The expression levels of activating Ly49D receptors were significantly increased (~30%) in the splenic NK cells of APNKO when compared with cells from WT mice (Fig. 3A). In the splenic NK cells, no significant differences in the expression levels of other receptors were identified between the APNKO and WT mice. To determine whether IL-2 further increased Ly49D expression on the NK cells from APNKO mice, we cultured splenocytes with IL-2 for 24 h. Under IL-2 stimulation, the proportion of Ly49D+ NK cells was significantly higher in the APNKO mice than in the WT mice (Fig. 3B, 3C), indicating that APN modulates NK cell activity by regulating the NK cell receptor repertoire.

NK cells are generated from HSCs, and APN has been reported to be an HSC growth factor (24). Thus, the increased NK cell population and cytotoxicity in the APNKO mice might be due to the altered differentiation of NK cells from the HSCs. To determine the exogenous role of APN in NK cell development, AdipoR1/R2 and T-cadherin mRNA expression levels were measured in HSCs that were isolated from WT and APNKO mice; however, HSCs expressed only AdipoR1/R2 (Fig. 4A). The isolated HSCs were cultured to stimulate differentiation to mNK cells, and mNK cell frequencies were then determined by flow cytometric analysis at 12 d after treatment. The differentiation of HSCs into mNK cells was significantly decreased in APNKO mice but was partially recovered by the addition of rAPN (Fig. 4B). Because activating Ly49D receptor expression levels were increased in NK cells from APNKO mice (Fig. 3A), APN might regulate Ly49D expression during NK cell development. As expected, the expression of Ly49D on mNK cells was suppressed by treatment with exogenous rAPN (Fig. 4C).

Reduced splenic MDSC accumulation in EL4 cell–challenged APNKO mice

We evaluated the MDSC (CD11b+Gr1+) populations in the spleens of EL4 tumor–bearing mice to determine whether MDSCs contributed to the enhanced anti-EL4 immunity in APNKO mice. We
found that the MDSC population in tumor-challenged APNKO mice was reduced by 50% when compared with the WT mice (Fig. 5A), despite similar splenic weights (Fig. 5B). Moreover, significant differences were observed in the MDSC subpopulations. The CD11b+Ly-6G+Ly-6Clow granulocytic MDSC (G-MDSC) and CD11b+Ly-6G+Ly-6Cint populations, although not the CD11b+Ly-6GlowLy-6C high monocytic MDSC (M-MDSC) population, were reduced in the EL4 cell–challenged APNKO mice (Fig. 5C). Additionally, the overall CD11b+ population was decreased in the EL4 cell–challenged APNKO mice. These findings indicate that the reduced accumulation of specific MDSC subpopulations in the spleens of APNKO mice under tumor-bearing conditions contributes to enhanced anti-EL4 immunity.

To determine whether APN was involved in MDSC differentiation and accumulation, we differentiated bone marrow–derived cells from APNKO and WT mice into MDSCs. To determine whether APN affects MDSC differentiation, rAPN was added to the cultures for 5 d during the ex vivo MDSC differentiation protocol. Bone marrow–derived cells from APNKO mice were less differentiated to the CD11b+ myeloid lineage cells than were the cells from WT mice (Fig. 5D), and the impaired differentiation to both MDSC populations in the APNKO mouse bone marrow was recovered by rAPN treatment (Fig. 5E). Next, we determined whether APN could regulate the immunosuppressive activities of MDSCs by measuring the expression levels of iNOS mRNA; iNOS, which is produced by MDSCs, can suppress T cells by various mechanisms (25). iNOS mRNA expression levels were downregulated in MDSCs that were differentiated from APNKO cells without rAPN treatment. These findings indicate that APN deficiency affects the immunosuppressive capacity of MDSCs (Fig. 5F).

G-CSF deficiency contributes to the decreased MDSC differentiation in APNKO mice

To determine whether APN regulated the expression of MDSC differentiation factors, we evaluated an inflammatory cytokine profile of serum from EL4 cell–challenged APNKO and WT mice by measuring the expression levels of iNOS mRNA; iNOS, which is produced by MDSCs, can suppress T cells by various mechanisms (25). iNOS mRNA expression levels were downregulated in MDSCs that were differentiated from APNKO cells without rAPN treatment. These findings indicate that APN deficiency affects the immunosuppressive capacity of MDSCs (Fig. 5F).
APN modulates NK cells, CTLs, and MDSCs

MHC class I molecule-upregulated B16F10 melanoma growth is retarded in APNKO mice

APNKO mice suppressed the growth of EL4 lymphomas. However, the growth of B16F10 melanoma cells was previously determined to be enhanced when tumor cells were s.c. injected into APNKO mice (10), and we confirmed this finding in our experimental conditions (Fig. 7A). To determine why APNKO mice present with such different immune responses to EL4 and B16F10, we measured the MHC class I (MHC I) expression levels on B16F10 and EL4 cells. EL4 cells expressed higher MHC I levels than did B16F10 cells (Fig. 7B). To determine whether this difference in MHC I expression could induce the different immune responses, B16F10 melanoma cells were treated with IFN-γ to increase the MHC I expression levels. IFN-γ treatment significantly increased the levels of MHC I expression on B16F10 cells, and the increased expression level was sustained for 10 d after the removal of IFN-γ (Fig. 7C). Notably, the growth of IFN-γ–treated B16F10 melanoma cells was significantly suppressed in APNKO mice (Fig. 7D, 7E). IFN-γ–treated B16F10 melanoma–bearing APNKO mice had large splenic CD3−CD4−CD8− T cell populations (Fig. 7F). However, the Ly49D receptor–expressing splenic NK cell population was greater in IFN-γ–treated B16F10 melanoma–bearing APNKO mice than in vehicle-treated B16F10 melanoma–bearing APNKO mice, although the splenic NK cell populations did not differ between the two groups (Fig. 7G).

Discussion

Previously, we demonstrated that APN inhibited NK cytotoxicity in vitro. In this study, we challenged APNKO mice with EL4 lymphoma cells to confirm our previous finding in vivo. As expected, EL4 tumor growth was suppressed in the APNKO mice, which was accompanied by increases in the NK cell population and activity. These data support our previous findings regarding the negative regulation of NK activity by APN. However, the in vitro differentiation of NK cells from HSCs was lower in the cells isolated from APNKO mice than from WT mice. There are two possible explanations for this finding. First, the increased NK population in APNKO mice might be due to increased NK cell trafficking from the bone marrow to the spleen in APNKO mice. Second, the HSCs isolated from APNKO mice might require different NK differentiation conditions in vitro, and the culture conditions in our experiments led to reduced NK cell differentiation. However, differentiated mNK cells from APNKO HSCs had increased expression levels of the Ly49D NK activation receptor, which were consistent with the levels of Ly49D expression on isolated NK cells. Moreover, the growth of EL4 cells in the APNHZ mice was highly suppressed, despite the modestly increased NK cell population, when compared with APNKO mice. When we measured the Ly49D+ NK cell population among total NK cells, the APNHZ mice had as many Ly49D+ cells as did the APNKO mice. These findings suggest that the low level of APN was sufficient to inhibit the growth of lymphoma cells via the increased NK cell sensitivity to the target cells (Supplemental Fig. 1C, 1D).

Ly49 proteins are NK cell receptors that recognize MHC I molecules and direct NK cells to recognize self- or non-self cells (26). Some of these receptors, including Ly49A, Ly49C, Ly49G2, and Ly49I, contain a cytoplasmic ITIM that inhibits the lysis of target cells (27, 28). Ly49D, in contrast, lacks this inhibitory motif, and the cross-linking of Ly49D activates NK cell cytolytic activity through intracellular kinase activation and calcium mobilization (29). Ly49D delivers signals to stimulate target cell lysis after interacting with the class I molecules H-2D6, D7, and D8.
EL4 tumor growth in APNKO mice. We determined the expression of Ag-specific antitumor CTLs (35). Thus, NK cells might activate Additionally, infiltrating NK cells play a key role in the generation cavities of mice, EL4 cells were cleared within 1 d of injection. Thus, we assume that the early onset of NK cell activity contributed to the delayed onset of EL4 lymphoma in APNKO mice. NK cells play a critical role in the killing of tumor cells in early-stage disease. When EL4 cells were injected into the peritoneal cavities of mice, EL4 cells were cleared within 1 d of injection. However, Ly49D can be stimulated by IL-12 and IL-18 and its cognate ligand (31). Additionally, NK cells can reject tumor cells that express ligands for activating NK cell receptors, despite the expression of MHC I on tumor cells (32). Unknown NK ligands might be involved in the APNKO-mediated generation of NK cells, or the environment in APNKO mice might stimulate ligand expression on EL4 tumors to activate the NK receptors. We also found that CTLs and NK cells contributed to the improved EL4 tumor growth regression in APNKO mice. Although we found no differences in the CTL populations from naive APNKO and WT mice, the CTL population was increased in EL4 tumor–bearing APNKO mice and was reduced by NK cell depletion. Meanwhile, IFN-γ is known to be a positive regulator of CTL activation (33), and the IFN-γ levels were higher in EL4 tumor–bearing APNKO mice than in WT mice (Supplemental Fig. 3A). Therefore, increased serum IFN-γ levels might promote increased CTL activity in APNKO mice.

The predominant tumor-infiltrating immune cells were T cells (53%), B cell lineage cells (33%), and macrophages (13%). NK and mature dendritic cells were rarely present (34). Nonetheless, NK cells play a critical role in the killing of tumor cells in early-stage disease. When EL4 cells were injected into the peritoneal cavities of mice, EL4 cells were cleared within 1 d of injection. Thus, we assume that the early onset of NK cell activity contributed to the delayed onset of EL4 lymphoma in APNKO mice. Additionally, infiltrating NK cells play a key role in the generation of Ag-specific antitumor CTLs (35). Thus, NK cells might activate anti-EL4–specific CTLs, leading to the inhibition of late-stage EL4 tumor growth in APNKO mice. We determined the expression levels of the Ly49D receptor on CTLs (Supplemental Fig. 3B) because Ly49D can activate CD8+ T cells through a TCR-independent mechanism (36). As expected, the expression levels of Ly49D-activating receptors were significantly higher on CTLs from APNKO mice, and these increased levels suggest a possible mechanism for the suppression of EL4 tumor growth in APNKO mice.

MDSCs act as immune suppressors through the secretion of immunosuppressive mediators such as IL-10, reactive oxygen species, and iNOS (37–39). In this study, MDSC accumulation was reduced in the spleens of tumor-bearing APNKO mice when compared with tumor-bearing WT mice. APN also facilitated the differentiation and enhanced immunosuppressive activity of MDSCs by upregulating iNOS transcription levels. Additionally, we showed that the decreased G-CSF secretion in EL4 tumor–bearing APNKO mice was related to the decreased MDSC differentiation in these mice. The mechanism by which G-CSF secretion was downregulated in APNKO mice remains to be determined. Meanwhile, it was revealed that globular APN robustly increased the secretion of G-CSF through the MEK1/2-ERK1/2 signaling pathway in RAW 264.7 cells (30). Therefore, it is unlikely that the upregulated expression of Ly49D receptors in APNKO mice affects NK cell–mediated antitumor immunity because both EL4 and B16F10 cells originate from C57BL/6 mice and express H-2Db. However, Ly49D can be stimulated by IL-12 and IL-18 and its cognate ligand (31). Additionally, NK cells can reject tumor cells that express ligands for activating NK cell receptors, despite the expression of MHC I on tumor cells (32). Unknown NK ligands might be involved in the APNKO-mediated generation of NK cells, or the environment in APNKO mice might stimulate ligand expression on EL4 tumors to activate the NK receptors. We also found that CTLs and NK cells contributed to the improved EL4 tumor growth regression in APNKO mice. Although we found no differences in the CTL populations from naive APNKO and WT mice, the CTL population was increased in EL4 tumor–bearing APNKO mice and was reduced by NK cell depletion. Meanwhile, IFN-γ is known to be a positive regulator of CTL activation (33), and the IFN-γ levels were higher in EL4 tumor–bearing APNKO mice than in WT mice (Supplemental Fig. 3A). Therefore, increased serum IFN-γ levels might promote increased CTL activity in APNKO mice.

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In a previous study, B16F10 melanoma cells grew rapidly in APNKO mice and were not directly inhibited by rAPN (10). This rapid growth was explained by the reduced recruitment of macrophages to the tumor sites. Peritoneal macrophages were elicited and co-implanted with B16F10 cells to determine whether a lack of tumor-infiltrating macrophages affected tumor growth. However, the elicited peritoneal macrophages were already activated
and had different gene expression profiles than tumor-infiltrating macrophages. Thus, other mechanisms cannot be ruled out.

To determine why APNKO mice respond differently to EL4 and B16F10, we evaluated the IL-2 mRNA expression levels because IL-2–secreting EL4 cells can enhance NK cell– and CTL-mediated innate immunity in tumor-bearing hosts (42). IL-2 mRNA expression was not observed in either the EL4 or the B16F10 cells (Supplemental Fig. 2C). Meanwhile, B16F10 and Lewis lung carcinoma cells were reported not to activate CTLs owing to low levels of MHC I expression (43); however, EL4 cells are generally used as CTL targets for in vitro CTL cytolytic assays because EL4 cells express high levels of MHC I. In this study, when MHC I expression levels were upregulated in B16F10 cells by IFN-α prior to injection into APNKO mice, the growth of these

FIGURE 5. Effects of APN on MDSC expansion, accumulation, and immunosuppression. (A–C) Splenic MDSCs (Gr1+CD11b+) were analyzed. The circle indicates the MDSC population among the total splenocytes. The numbers in each quadrant represent the percentages of gated cells (A, left), and the percentages of MDSCs are shown in a bar graph (A, right). The weights of spleens from EL4 tumor–bearing WT or APNKO mice were measured (B). M-MDSCs (CD11b+Ly6G−Ly6Chigh), G-MDSCs (CD11b+Ly6G1Ly6Chigh), and CD11b+Ly6G1Ly6C− (Int) cells were analyzed (C). The numbers in each quadrant represent the percentages of gated cells. The results are expressed as the means ± SD of three separate experiments (n = 5). (D–F) An ex vivo MDSC differentiation assay was performed. CD11b+ cells (D) and MDSC subpopulations (E) were measured after 5 d in culture. (F) iNOS mRNA expression levels in the differentiated MDSCs were analyzed by RT-PCR. The results are expressed as the means ± SD of three separate experiments (n = 3). *p < 0.05, **p < 0.01 for WT versus APNKO mice, or the two indicated groups; age-matched C57BL/6J mice were used as WT controls. A one-way factorial ANOVA was used for the comparison of multiple groups.

FIGURE 6. Low G-CSF levels in EL4 cell–challenged APNKO mice and increased expansion of MDSCs in APNKO bone marrow cells after exogenous G-CSF treatment. (A) G-CSF levels were measured in the sera from EL4 tumor–bearing WT and APNKO mice with an ELISA-based cytokine array panel (A, right). The densities were measured and are expressed as fold increases (A, left). An ex vivo MDSC differentiation assay was performed. G-MDSCs (CD11b+Ly6G−Ly6Chigh; B) and M-MDSCs (CD11b+Ly6G−Ly6Chigh; C) were measured after 5 d in culture. The results are expressed as the means ± SD of two separate experiments (n = 3). **p < 0.01, ***p < 0.001 for WT mice versus APNKO mice; age-matched C57BL/6J mice were used as WT controls. A PBS-based 0.1% BSA solution was used as the vehicle control.
enforced MHC I–expressing B16F10 cells was retarded by an increased CTL population. Therefore, varied MHC I molecule expression levels on the tumor cells account for the different growth responses that were observed in APNKO mice. Moreover, the increased expression of MHC I molecules on the tumor cells induces the activation of effector cells and facilitates the transition from innate immune responses to more efficient adaptive antitumor immune responses (44).

We examined the expression levels of the three known APN receptors, including AdipoR1/R2 and T-cadherin. We found that NK cells from WT and APNKO mice expressed similar levels of AdipoR1/R2 and similarly low levels of T-cadherin. These data indicate that the effects of APN on NK cells are not mediated by differences in receptor expression levels. However, the levels of APN and the T-cadherin receptor are not associated with APN-mediated NK cell function. We also analyzed the levels of the three APN receptors in MDSCs. MDSCs from the APNKO mice expressed low levels of the three APN receptors. However, owing to the lack of APN in APNKO mice, the low receptor expression levels are unlikely to correlate with the effects of APN on MDSC function. Meanwhile, it was reported that TCR engagement upregulates AdipoR1/R2 on the surfaces of T cells but that APN negatively regulates the proliferation and function of Ag-specific CD8+ T cells (45). These findings suggest that APN suppresses CTL activity through binding to AdipoR1/R2. However, T-cadherin receptor expression was not observed in an analyzed spleen, although 47% of the splenocytes were T cells (Supplemental Fig. 2D–F).

In summary, APN directly inhibits the growth of breast and colorectal tumor cell lines (22, 46, 47), and tumors that are generated or injected in APNKO mice grow rapidly. Although this rapid tumor growth in APNKO mice was reported previously, a detailed in vivo molecular mechanism was elusive. Interestingly, EL4 lymphoma cells grew slowly in APNKO mice, unlike other tumor cell lines. We demonstrated that the absence of APN generates changes in the antitumor immune cell populations, including NK cells, CTLs, and MDSCs. These changes permitted the slow growth of EL4 lymphoma cells in APNKO mice. Specifically, the surface expression levels of MHC I on the tumor cells were critical factors in the determination of tumor growth rates in APNKO mice. The growth rate of MHC Ihigh B16F10 melanomas in APNKO mice is retarded compared with MHC Ilow B16F10 melanomas. (A) Tumor volume changes and weights in APNKO mice. Mice (n = 5) were s.c. injected with 2 × 10⁵ B16F10 cells; the tumor volumes were measured up to 21 d after tumor injection, at which time the tumor weights were measured. (B) The expression level of MHC I (H-2Kb/H-2Db+) was measured (n = 3). (C) B16F10 cells were treated with recombinant IFN-γ. The expression level of MHC I (H-2Kb/H-2Db+) was measured on alternating days (n = 3). (D–G) After 24 h IFN-γ treatment, the MHC Ihigh B16F10 cells were collected and s.c. injected into APNKO mice (n = 3). Tumor growth was monitored by measuring changes in volume (D), and tumor weights were measured on the final day (E). Splenic CTLs (CD3⁺CD8⁺CD4⁻; F), splenic NK cells (NK1.1⁺CD3⁺; G, left), and the expression level of Ly49D on NK cells (Ly49D⁺CD3⁺NK1.1⁺; G, right) were measured. *p < 0.05, **p < 0.01 for WT mice versus APNKO mice or vehicle-treated B16 tumor-bearing APNKO mice (B16-Veh) versus IFN-γ-treated B16 tumor-bearing APNKO mice (B16-IFN-γ). Age-matched C57BL/6J mice were used as WT controls.
Supplemental Figure 1. Effects of APN on the anti-tumor activity in female and obese mice. (A) Tumor volume growth curves and weights in APNKo mice. Mice were s.c. injected with EL4 cells. Results are expressed as the means ± SD (n=5). *, p < 0.05; **, p < 0.01 for WT male versus female mice. (B) WT, APN heterozygote (HZ), db/db obese (DB) and APNKo (KO) mice were s.c. injected with EL4 cells. Splenic NK cell (NK1.1+CD3-) (C) and Ly49D-expressing NK cell (Ly49D+/NK1.1+CD3-) populations (D) were measured in individual naïve mice. Results are expressed as the means ± SD (n=4). *, p < 0.05; **, p < 0.01 between the indicated two groups. Statistical significance was analyzed with a one-way factorial ANOVA.
Supplemental Figure 2. *In vitro* effects of APN on B16F10 and EL4 cell proliferation and T-cadherin mRNA expression in immune cells *in vivo.* (A) *In vitro* proliferation assay. A total of 2 x 10⁵ B16F10 or EL4 cells were seeded onto 12-well plates, and cell viability was determined by trypan blue staining at 24 h post-rAPN treatment. Results are expressed as the means ± SD of triplicate experiments (n=3). (B) AdipoR1/R2 and T-cadherin mRNA expression in B16F10 and EL4 cells. (C) IL-2 mRNA expression in B16F10 and EL4 cells was analyzed by RT-PCR. (D) T-cadherin mRNA expression in various tissues from normal mice was analyzed by RT-PCR. (E-F) T-cadherin mRNA expression in isolated splenic NK cells from naïve WT or APNKO mice and isolated splenic MDSCs from EL4-bearing WT or APNKO mice was analyzed by RT-PCR.
Supplemental Figure 3. Increased frequencies of Ly49D-expressing CTLs in tumor-bearing APNKO mice. (A) Serum was obtained from EL4 tumor-bearing WT and APNKO mice, and serum IFN-γ levels was analyzed with an ELISA-based cytokine array panel. (B) Freshly isolated splenocytes from individual WT or APNKO mice were stained with fluorescent-conjugated antibodies to analyze the Ly49D-expressing CTL population (CD3+CD4−CD8+Ly49D+). All results are expressed as the means ± SD of three separate experiments (n=3). Shown are dot plots of analyzed cells; the numbers in each box represent the percentages of cells. * p < 0.05 for WT versus APNKO mice. Age-matched C57BL/6J mice were used for WT controls.
Supplemental Table I. The percent of lymphocyte population (% of the total number of cells)\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Naïve</th>
<th>EL4-tumor-bearing</th>
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<tbody>
<tr>
<td></td>
<td>Spleen</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>NK cells</td>
<td>4.4 ± 0.1</td>
<td>5.6 ± 0.3*</td>
</tr>
<tr>
<td>NKT cells</td>
<td>8.6 ± 0.9</td>
<td>5.8 ± 0.3*</td>
</tr>
<tr>
<td>CTL</td>
<td>5.4 ± 0.3</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Treg</td>
<td>5.2 ± 0.7</td>
<td>3.7 ± 0.3</td>
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\textsuperscript{a} Mice were s.c. injected with 2 x 10^5 cell numbers of EL4 cells. 21 days after tumor freshly isolated splenocytes and bone marrow cells from individual WT or APNKO mice under naïve or tumor-challenged conditions were stained with combinations of antibodies to analyze NK cell (NK1.1+CD3-), NKT cell (NK1.1+CD3+), CTL (CD3+CD8+CD4-) and Treg (CD4+CD25+) populations. The percent (%) of immune cells were determined by calculating the absolute numbers of each cell type from the FACS profiles and by the total number of cells. Results are expressed as mean ±S.D of three separate experiments (n=3~5). *p<0.05, **p<0.01, ***p<0.001 for WT versus APNKO mice. Age-matched C57BL/6J was used for WT controls versus APNKO mice.