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Research Article

Prevention of Spontaneous Tumor Development in a *ret* Transgenic Mouse Model by Ret Peptide Vaccination with Indoleamine 2,3-Dioxygenase Inhibitor 1-Methyl Tryptophan

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Abstract

The present study investigated an immunotherapeutic strategy for rearranged during transfection proto-oncogene (*ret*)–associated carcinomas in a transgenic MT/*ret* 304/B6 mouse model in which spontaneous tumors develop due to overexpression of the *ret* gene. A Ret peptide vaccine comprising an extracellular fragment of Ret protein and Th1-polarized immunoregulator CpG oligonucleotide (1826) induced strong and specific cellular and humoral immune responses in wild-type C57BL/6 mice, showing that the Ret peptide has a strong immunogenic potential as part of an antitumor vaccine. In MT/*ret* 304/B6 mice, however, the vaccine was only modestly effective as an inducer of the humoral immune response, and it failed to elicit a T-cell response. An immunohistochemical analysis revealed marked indoleamine 2,3-dioxygenase expression after immunization with Ret peptide vaccine in the lymph nodes and spleens of MT/*ret* 304/B6 mice. The systemic administration of the potent inhibitor of indoleamine 2,3-dioxygenase 1-methyl tryptophan (1MT) along with Ret vaccine produced a significant increase in tumor-specific cytotoxic activity. A delay in spontaneous tumor development was also observed in the MT/*ret* 304/B6 mice to which the Ret vaccine and 1MT were administered. These results indicate that an improved Ret vaccine composed of Ret peptide plus CpG oligonucleotide plus 1MT is a potential therapeutic strategy for treatment of *ret*-associated carcinomas. [Cancer Res 2009;69(9):3963–70]

Introduction

The expression of Ret-receptor tyrosine kinase (1) is necessary for the development of neuroendocrine system (2), kidneys (3), regulation of thyroid function (4, 5), and spermatogenesis (6), whereas the mutations of the rearranged during transfection (*ret*) gene could induce Ret-receptor tyrosine kinase abnormal activation and accordingly initiate several neuroendocrine tumors, such as multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma (7–10). Medullary thyroid cancer is the dominant endocrinopathy (frequency >95%) in patients with MEN or familial medullary thyroid carcinoma, which exhibits poor prognosis and high mortality (11). Although it is speculated that the development of inhibitors against Ret-receptor tyrosine kinase signaling may be beneficial to the desired clinical outputs, most cases of medullary thyroid cancer do not have known mutations, and no single *ret* inhibitor proves to be effective in all cases (12). Therefore, it is very significant to explore the immunotherapy targeting Ret protein to improve the clinical outputs for conquering *ret*-associated carcinomas.

Tumor immune escape has become a bottleneck in cancer immunotherapy, and numerous associated mechanisms have been implicated. Among them, the indoleamine 2,3-dioxygenase (IDO) mechanism has been regarded as focus of study, because IDO activities are commonly accompanied by the emergence of tumors (13). Recent data indicate that IDO creates a profoundly immunosuppressive microenvironment within the tumor site and/or tumor-draining lymph nodes (TDLN) via the activities of the antigen-presenting cells, especially the plasmacytoid dendritic cells (14, 15). Regulatory T cells can induce a high level of functional IDO in mouse dendritic cells (16). By depleting tryptophan and producing activated metabolites, IDO inhibits the proliferation and activation of antigen-specific T lymphocytes and conducts tolerance (17, 18). Moreover, IDO expression in the antigen-presenting cells can be up-regulated by the cancer treatments themselves, such as chemotherapeutic drugs, tumor vaccines, and immunostimulants, including IFN-γ, IFN-α, CpG oligodeoxynucleotides, 4-1BB ligation (19–23), etc. Thus, to effectively inhibit the IDO pathway may contribute to breaking the immune tolerance to tumors to make advances in immunotherapy and chemotherapy (24–27).

Here, we explored the immunotherapeutic strategy for *ret*-associated carcinomas by using a transgenic mouse line, MT/*ret* 304/B6 mice, which contained the human *ret* proto-oncogene. Due to the high expression of Ret protein, the mice develop *ret*-associated tumors spontaneously (28, 29). Following administration with Ret peptide plus Th1-polarized immunoregulator CpG oligonucleotide, the mice developed a strong humoral immune responses but not T-cell response. However, simultaneously administered with 1-methyl tryptophan (1MT), a potent inhibitor of IDO, a strong specific T-cell response and a delayed spontaneous tumor development were observed. These findings suggest a potential immunotherapeutic strategy for treating *ret*-associated carcinomas.

Materials and Methods

Animals. C57BL/6 mice were purchased from Sun Yat-sen University. MT/*ret* transgenic 304/B6 male seed mice were presented by the Immunology Department of the Medicine School of Nagoya University.

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All animals were kept under standard pathogen-free conditions and MT/*ret* 304/B6 seed mice were backcrossed twice with C57BL/6 mice; the newborn MT/*ret* 304/B6 transgenic mice (with all-black skin at 5 days after birth; ref. 28) were raised to age 3 weeks and immunized in this study. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals.

Media and reagents. RPMI 1640 (Life Technologies) supplemented with 5% or 10% fetal bovine serum was used to culture cells for CTL and ELISPOT assays. Purified anti-CD8 (Biolegend) and anti-IDO (Chemicon) were used to detect IDO and CD8 expression in tumors and immune organs by immunohistochemistry. The IDO inhibitor, DL-1MT, was purchased from Sigma. To prepare DL-1MT for oral gavage, 5 g DL-1MT was added to a 100 mL beaker with 42.5 mL Methocel/Tween (0.5% Tween 80/0.5% methylcellulose). The mixture was stirred overnight at 4°C. The next day, the DL-1MT concentration was adjusted to 80 mg/mL by adding an additional 20 mL Methocel/Tween and stirring continuously at 4°C. An *in vitro* study has been done to assess the inhibitory activity of DL-1MT on IDO, and results showed that DL-1MT could effectively inhibit the activity of IDO induced by IFN-γ in RAW264.7 cells. An 84.9% down-regulation was estimated by evaluating the concentration of kynurenine transformed from tryptophan in the cultural medium (data not shown).

Antigens and adjuvant. Recombinant human Ret (mRNA 190-807, M16029) antigen (with 6× histidine at the COOH-terminal, 25 kDa) was expressed via pET-28a(+) (Novagen) in BL21 and purified in our laboratory. All-phosphorothioate modified CpG oligonucleotide 1826 (5′-TCCAT-GACGTTCCTGACGTT-3′) was synthesized by the Shanghai Sangon Biological Engineering & Technology and Service.

Vaccine preparation. The Ret antigen and CpG oligonucleotide 1826 were, respectively, dissolved in PBS. Mixed two liquids to obtain a suspension including 1,500 μg (first vaccine) or 750 μg (boosts) Ret, with 150 μg CpG oligonucleotide 1826/mL. We defined this cocktail vaccine as Ret/CpG vaccine.

Immunizations and measurement of tumor growth. The normal C57BL/6 (4 weeks old) and MT/*ret* 304/B6 (3 weeks old) mice were immunized subcutaneously and multipointly on the back with 0.2 mL/mouse Ret/CpG vaccine, PBS, or PBS/CpG (Fig. 1). For preventive research, the age of initial vaccination was set at age 3 weeks because MT/*ret* 304/B6 mice grew subcutaneous tumors that were commonly found in the head and neck region after age 4 weeks (28). All mice were boosted twice with a half-dosage of Ret protein at 2 weeks (first boost) and 3 weeks (second boost) after the first vaccination. For inhibiting the activity of IDO, the DL-1MT slurry was administered by oral gavage at 400 mg/kg/dose, twice a day, from 5 days before the first vaccine until 3 days after the second boost. All MT/*ret* 304/B6 mice were checked weekly for whole-body palpable tumors. Tumor size was measured in diameter with a pair of Vernier calipers. The total tumors volume of each mouse was determined by the following formula: volume = $\Sigma 4/3\pi r^3$, where r represents the radius of each tumor.

Measurement of serum Ret antibody by ELISAs. The blood of each individual mouse was obtained by tail-end sampling, and serum was collected. The 96-well plates were precoated with Ret antigen (50 ng/well) and blocked with 3% bovine serum albumin for 1 h at room temperature. The serum samples were added in serial dilutions at a gradient from 1:800 to 1:102,600. Plates were then washed, and horseradish peroxidase-conjugated anti-IgG (1:5,000; Boster) was added for 1 h at room temperature. After being washed, the plates were developed with tetramethylbenzidine and hydrogen peroxide and read at A_{405} using an Elx800 universal microplate reader (Bio-Tek).

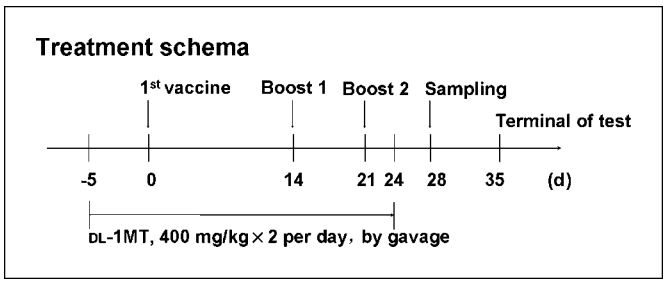

Figure 1. Schematic representation of the treatment schedule and dosages in mice.

Measurement of specific antibody by Western blotting. The Ret antigen/homogenated tumor tissue from MT/*ret* 304/B6 mice was run on 8% SDS-PAGE, and the proteins were blotted onto a polyvinylidene fluoride membrane. The membrane with the diluted serum (1:1,000 in PBS-Tween 20) from each individual mouse was then incubated. Horseradish peroxidase-conjugated anti-mouse IgG (1:5,000; Boster) was then incubated to detect the specific anti-Ret signals via the ECL Western blotting method (Santa Cruz Biotechnology).

Immunohistochemical analysis for IDO and CD8 expression in tumors and immune organs. Some mice from each group were sacrificed 1 week after the second boost, and the cervical lymph nodes (in MT/*ret* 304/B6 mice, tumors were always earliest found in the head and neck), spleen, and tumors from individual mice were harvested. The IDO and CD8 were detected by immunohistochemical assays. The tumors and lymphoid organs were fixed in formalin and embedded in paraffin, and the paraffin blocks were sectioned. Sections were deparaffinized and then incubated overnight at 4°C with monoclonal antibodies at a dilution 1:50 for the detection of IDO and 1:100 for the detection of CD8. After three washes with wash buffer containing 0.02% Tween 20, slides were incubated with the horseradish peroxidase-labeled polymer-conjugated secondary antibody (EnVision+; DAKO), and DAB (DAKO) was applied as a substrate. The slides were rinsed in distilled water and counterstained with hematoxylin (Sigma-Aldrich).

The staining intensity of IDO or CD8 was also confirmed using the National Center for Biotechnology Information ImageJ software. Image analysis was done to quantify the intensity of IDO or CD8 staining. The acquiring image was captured at ×200 or ×400 magnification. A color threshold for brown (IDO positive or $CD8^+$) and blue (nuclear stain) staining was set for every slide analyzed. Thirty to 50 regions of interest (radius of each region, 60 μm) were manually selected and each selected region was individually calculated for the percentage of positive cells. The average percentage of positive cells for all regions was calculated as the mean ± SE for statistical comparison.

Assessment of CTL assays. A primary cultured cell, Melan-*ret* cell, as described in the literature (30), was established from a cervical nodule of a MT/*ret* 304/B6 mouse by collagenase (type II) digestion for subsequent use as a target cell in the CTL assays. Melan-*ret* cell was cultured for 24 h in complete medium containing 100 units/mL mouse IFN-γ (PeproTech) to significantly enhance MHC expression, thereby increasing the probability of being recognized by specific T cells. One week after the second boost, spleens were removed from mice for the preparation of single-cell suspensions by pressing against fine nylon mesh. Red cells were lysed using 0.84% ammonium chloride. The splenic cells from individual mice were cultured for 24 h in complete medium containing IL-2 at 300 units/mL (PeproTech). Spleen lymphocytes were cocultured with primary cultured Melan-*ret* cell at 25:1, 50:1, and 100:1 in RPMI 1640 plus 10% fetal bovine serum. Five days later, cytotoxicity assay was conducted by using a nonradioactive lactate dehydrogenase early releasing detection kit (CytoTox 96; Promega) as indicated in the manufacturer's instructions. The spontaneous release and maximum release were determined by incubating target cells without effector cells in medium alone or in 0.5% NP-40, respectively. The percent cytotoxicity was calculated as follows: (experimental release - spontaneous release) / (maximum release - spontaneous release) × 100%.

Assessment of Ret antigen–specific IFN-γ–producing cells by ELISPOT. IFN-γ–producing cells were assessed by a specific ELISPOT set (DAKEWE Biotech) according to the manufacturer's protocol. In brief, single-cell suspensions were prepared from spleens 1 week after the second boost and plated in 96-well microplates that have been precoated with anti–IFN-γ antibody specific for ELISPOT. Cells were incubated for 48 h in the presence or absence of Ret antigen (2 μg/mL) plus human IL-2 (300 units/mL). Horseradish peroxidase-conjugated anti-mouse IFN-γ antibody was added, developed with ready-to-use amino-9-ethyl carbazole, and read by a Bioreader 5000 (Bio-Sys).

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Statistical analysis. A statistical evaluation of the differences between the means of the experimental groups was done by analyzing variance and performing a nonparametric two-tailed t test. $P < 0.05$ was considered significant.

Results

Ret peptide vaccine induced strongly specific immune response in wild-type C57BL/6 mice. To determine whether vaccination against Ret engenders an immune response, we first performed a test to analyze specific anti-Ret IgG and specific cellular immune response in wild-type C57BL/6 mice immunized with an extracellular fragment of Ret peptide alone. Seven days after the last boost, only low levels of specific antibodies and low CTL responses were detected in the mice immunized with the Ret peptide alone (Fig. 2) in spite of a strong specific humoral immune response that was observed in mice immunized with the unrelated antigen OVA (data not shown). Because CpG oligonucleotide 1826 was described as a potential adjuvant when coadministered with polypeptide (31, 32) and effectively enhanced the cellular immunologic response, a vaccine comprising Ret protein and CpG oligonucleotide 1826 (Ret/CpG) was used to elicit immune responses.

As shown in Fig. 2*A*, compared with the mice immunized with Ret peptide alone, the Ret/CpG vaccine elicited a more significant humoral immune response (each group, $n = 8$; $P < 0.01$). This specific immune response in the mice was further shown by Western blotting using cell lysate from Ret-expressing Melan-*ret* cell. The results showed that the anti-Ret antibody from individual immunized mice could recognize Ret protein from the lysate of tumor tissue from MT/*ret* 304/B6 mice, whereas no bands appeared at the same site when using the serum from unprotected mice (Fig. 2*B*). These data indicated that the Ret/CpG vaccine elicited an antibody specific to the Ret antigen. CTL assay studies also showed a stronger Ret antigen specific to the CTL response in the Ret/CpG vaccine-immunized mice compared with the mice immunized with Ret peptide alone (Fig. 2*C*). Furthermore, the ELISPOT assays showed that the frequency of Ret-specific IFN-γ–producing cells had progressed. As shown in Fig. 2*D*, the cells from the spleens of Ret/CpG vaccine-immunized mice produced 210.6 ± 41.5 spots in 10^6 cells, whereas, in unprotected mice (those injected with PBS + CpG), the splenocytes produced only 11.9 ± 5.3 spots in 10^6 cells (each group, $n = 8$; $P < 0.01$). These results suggested that the immune responses induced by Ret peptide were augmented by a coadministration with CpG, a potential immune adjuvant, and that Ret/CpG would have potential as an effective vaccine in targeting the setting of *ret*-associated carcinoma immunotherapy.

Ret/CpG vaccine induced modestly humoral immune response but failed to produce effectual antitumor responses in the *ret* transgenic mice. To assess the preventive efficacy of the vaccine against *ret*-associated carcinoma, the Ret/CpG vaccine was administered to MT/*ret* 304/B6 mice with no tumor burden, and their immune responses were analyzed by ELISAs, CTL assays, and IFN-γ ELISPOT assays. As shown in Fig. 3*A*, following vaccination with the Ret/CpG vaccine alone, there was a dramatic increase in the amount of specific anti-Ret antibody, a response as strong as that in the Ret/CpG vaccine-immunized wild-type C57BL/6 mice ($P > 0.05$, compared with Fig. 2*A*). However, the CTL assays and ELISPOT IFN-γ assays showed that the Ret/CpG vaccine induced only a lower level cellular immune response ($P < 0.01$, compared with Fig. 2*C* and *D*). The specific lysis of the target cell (Melan-*ret* cell) by effector cells (splenic T cells) was 13.26 ± 5.96% (Fig. 3*B*), and the antigen-specific IFN-γ–releasing cells/10^6 splenocytes from transgenic mice immunized with Ret/CpG vaccine was 46.17 ± 11.79 (Fig. 3*C*). Consistent with this, the tumors reached a large size in the Ret/CpG vaccine-immunized mice, which was just weakly reduced compared with the control

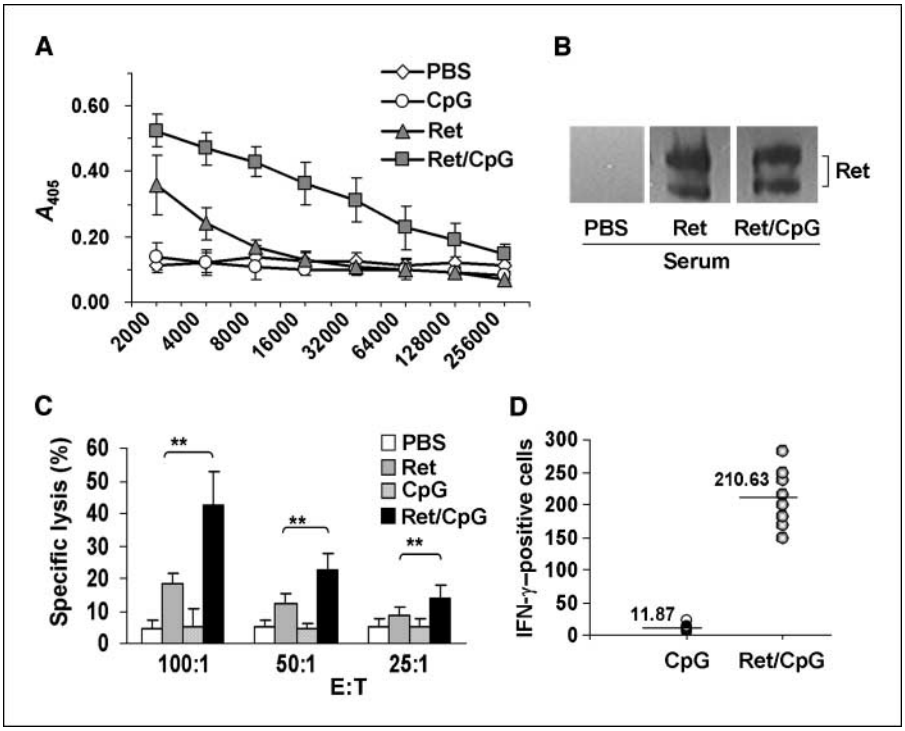

Figure 2. Effects of Ret vaccinations on immune responses in wild-type C57BL/6 mice. Mice were treated with vaccinations as described in Materials and Methods. One week after the second boost, the serum and splenocytes of individual mice were collected for immune response assessment (each data set in *A-D*; $n = 8$). *A,* ELISA results of serum anti-Ret antibody. The concentration of precoated Ret antigen was 50 ng/well. The color reaction of tetramethylbenzidine was read at A_{405}. *B,* Western blotting analysis of the serum anti-Ret antibody specificity. The antigen is the lysate of tumor tissue from MT/*ret* 304/B6 mice. *C,* tumor-specific CTL response analysis. The target cell is the primary cultured Melan-*ret* cell, collected from the tumor tissue of MT/*ret* 304/B6 mice, and the CTL responses to tumor cells were determined via a lactate dehydrogenase early release assay. **, $P < 0.01$. *D,* Ret-specific IFN-γ ELISPOT assays of splenocytes. Splenocytes were incubated for 48 h in the presence of Ret antigen (2 μg/mL) plus human IL-2 (300 units/mL), and the frequency of IFN-γ–positive cell production was assayed. Results are expressed as spots per 10^6 splenocytes.

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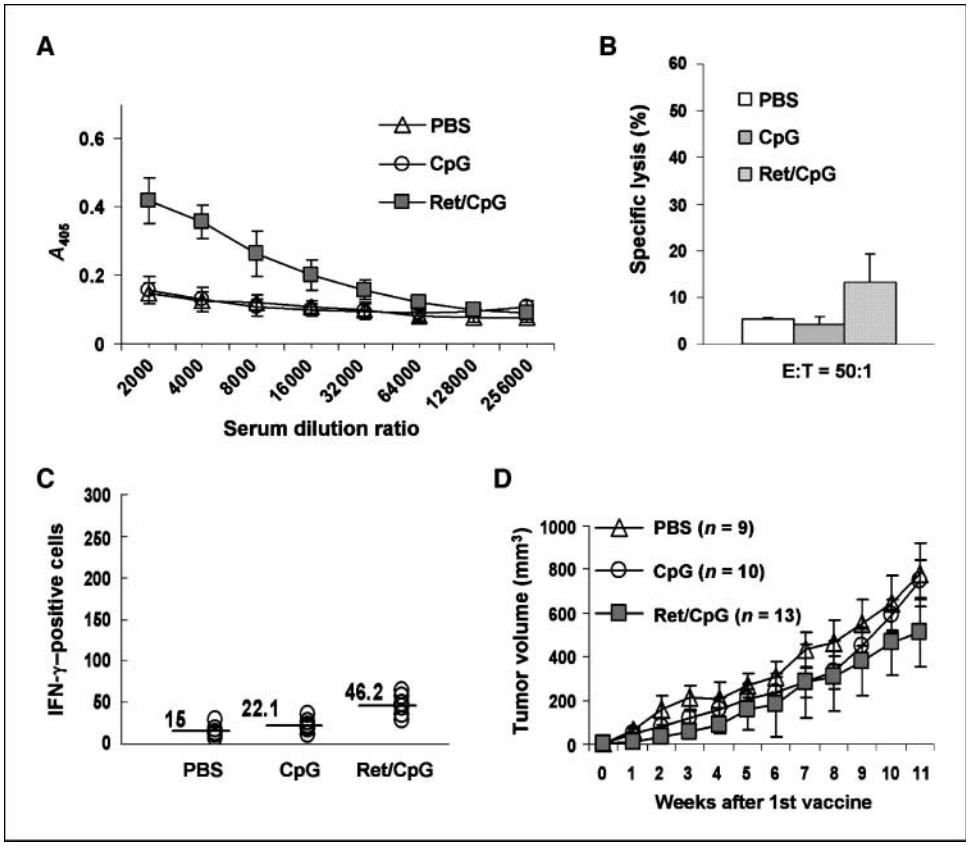

Figure 3. Effects of Ret/CpG vaccinations on immune responses and tumor growth in MT/*ret* 304/B6 mice. The mice were administered vaccinations as described in Materials and Methods (each data set in *A-C*; $n = 6$). *A,* ELISA results of serum anti-Ret antibody. *B,* tumor-specific CTL response analysis. *C,* Ret-specific IFN-γ ELISPOT assay of splenocytes. *D,* weekly tumor growth analysis ($n = 9$-13). Each mouse was vaccinated and tumors were measured as the mean ± SE once per week. The total tumor volume was determined using the formula: volume = $\Sigma 4/3\pi r^3$, where r represents the radius of each tumor.

mice (mean volume of tumors at 11 weeks after first vaccine, 510.62 ± 158.32 versus 777.48 ± 146.63 mm^3; $P < 0.01$; Fig. 3*D*). The mice were also treated daily with IFN-γ intraperitoneally during vaccination against Ret, and tumor progression was determined by measuring time to tumor appearance. Contrary to expectations, the results showed that treatment with Ret/CpG vaccine plus IFN-γ could not diminish the efficacy in protective tumor immunity of Ret/CpG vaccine [14.51 ± 3.01% in specific cytolysis ($n = 6$), 51.67 ± 8.99 in IFN-γ secretion ($n = 6$), and 494.46 ± 139.59 mm^3 in tumor progression ($n = 12$); data not shown in figures]. These results suggested that the Ret/CpG vaccine did induce specific humoral immunity but that it was not sufficient to elicit an effective antitumor immune response in MT/*ret* 304/B6 mice.

High level of IDO expression in lymph nodes and spleen in *ret* transgenic mice. Because the IDO pathway was believed to be one of the important mechanisms behind tumor immune tolerance by creating an immunosuppressive microenvironment within the tumor site and/or TDLNs and spleen, we performed immunohistochemical studies to verify whether IDO expresses in the tumor tissues and/or immune organs in MT/*ret* 304/B6 mice. The results showed that IDO expression was not found in the spleens and cervical lymph nodes in C57BL/6 mice (Fig. 4*A* and *B, a* and *b*), whereas high levels of IDO expression were found in the TDLNs and spleens, but not in tumor tissues, in tumor-bearing MT/*ret* 304/B6 mice (Fig. 4*A* and *B, c-e*). Importantly, the intensity of the IDO-positive cells was markedly increased in the spleen and TDLNs after being vaccinated with the Ret/CpG vaccine (average % of IDO-positive cells, 43.7 ± 12.6% versus 15.4 ± 4.2% in spleens, $P < 0.01$, and 55.3 ± 14.7% versus 24.5 ± 7.9% in TDLNs, $P < 0.01$; Fig. 4*A* and *B, f-h*). These results implied that the IDO-mediated immunosuppression might be involved in the immune tolerance to *ret*-associated tumors.

Ret/CpG vaccination with 1MT treatment develops specific immune responses in *ret* transgenic mice. 1MT was recently identified as a potent inhibitor of IDO, and it was successful in enhancing antitumor immunity in many tumor implantation animal models (33–35); we performed this experiment to determine whether 1MT could enhance the efficacy of the Ret/CpG vaccine in MT/*ret* 304/B6 mice. As shown in Fig. 5*A*, compared with the control group mice, the mice administered Ret/CpG alone sustained a remarkable increase in antibody production against the Ret antigen, but the level of antibody against the Ret antigen was not further enhanced in the mice that were coadministered Ret/CpG plus 1MT ($P > 0.05$), indicating that the inhibition of IDO activity had no effect on the humoral immune response.

T-cell responses were assessed by CTL assays and an IFN-γ ELISPOT assay. As shown in Fig. 5*B*, the tumor-specific CTL response in the Ret/CpG plus 1MT-treated mice was two times greater than in the mice treated with Ret/CpG alone (cytotoxicity, 27.20 ± 6.77% versus 13.26 ± 5.96%; E:T ratio, 50:1; $P < 0.01$). Accordingly, an IFN-γ ELISPOT assay also showed that the frequency of antigen-specific CTL cells was remarkably enhanced by the administration of 1MT in the Ret/CpG vaccine-immunized mice (positive spots in 10^6 splenic cells, 131.67 ± 23.91 versus 46.17 ± 11.79; $P < 0.01$; Fig. 5*C*). The 1MT-dependent increase in specific cytolysis action and IFN-γ secretion of CTL was further promoted by administration with IFN-γ (37.42 ± 9.92% in specific cytolysis and 183.33 ± 25.45 in IFN-γ secretion; each group, $n = 6$; data not shown in figures), whereas administration with IFN-γ alone did not obviously change these antitumor responses. Taken

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together, these results indicated that IDO was involved in the limitation of anti–self-antigen immune responses and that inhibiting the activation of IDO might be beneficial in repairing the potent Th1-polarized immune response against spontaneous tumors.

Synergistic effect of Ret/CpG vaccine and 1MT on delaying tumor growth. To determine whether the inhibition of IDO activity could restore the cellular immune unresponsiveness to Ret antigen *in vivo*, we performed immunohistochemical $CD8^+$ assays to assess the infiltration of lymphocytes into tumor tissues. As shown in Fig. 6*A* and *B*, 1MT treatment alone could not induce T-cell infiltration of tumors. The $CD8^+$ cells were rarely observed in the tumor sites in the mice after being immunized with the Ret/CpG vaccine alone, but the numbers of $CD8^+$ cells were obviously increased by the coadministration of Ret/CpG vaccine and 1MT (average % of $CD8^+$ cells, $2.2 \pm 0.6\%$ versus $0.5 \pm 0.1\%$; $P < 0.01$).

During immunization, tumor progression in individual mice in different groups was also checked, and the results of tumor progression were illustrated in Fig. 6*C*. 1MT treatment alone did not inhibit tumor growth. Compared with the weakly inhibited tumor progression in the mice that were treated with Ret/CpG vaccine alone, a significant delay in tumor development was observed in the mice that were coadministered the Ret/CpG vaccine with the IDO inhibitor, 1MT (mean volume of tumors at 11 weeks after first vaccine, 225.46 ± 106.76 versus 510.62 ± 158.32 mm^3; $P < 0.01$), and this effect was further enhanced by added IFN-γ treatment (111.24 ± 40.47 mm^3 at 11 weeks; $n = 11$; $P < 0.01$; data not shown in figures). These results suggested that an IDO-mediated mechanism was involved in inhibiting the cellular immune response to the Ret vaccine and that a vaccine comprising the Ret peptide plus CpG oligonucleotide plus 1MT was a potentially efficient tool in eliminating immune tolerance with regards to *ret*-associated spontaneous tumors.

Discussion

A main criterion in choosing candidate self-antigens for cancer immunotherapy is that the antigens are nonmutated gene products or "universal" fragments that are suitable to the majority of patients. To date, there has been no evidence of genetic mutations in Ret (amino acids 64-269), the antigen component we chose in the vaccine, in human *ret*-associated carcinomas. In addition, many potential T-cell epitopes were found in this segment by prediction of GENETYX-MAC software. It is therefore reasonable to design a vaccine based on $Ret_{64\text{-}269}$ to stimulate protective

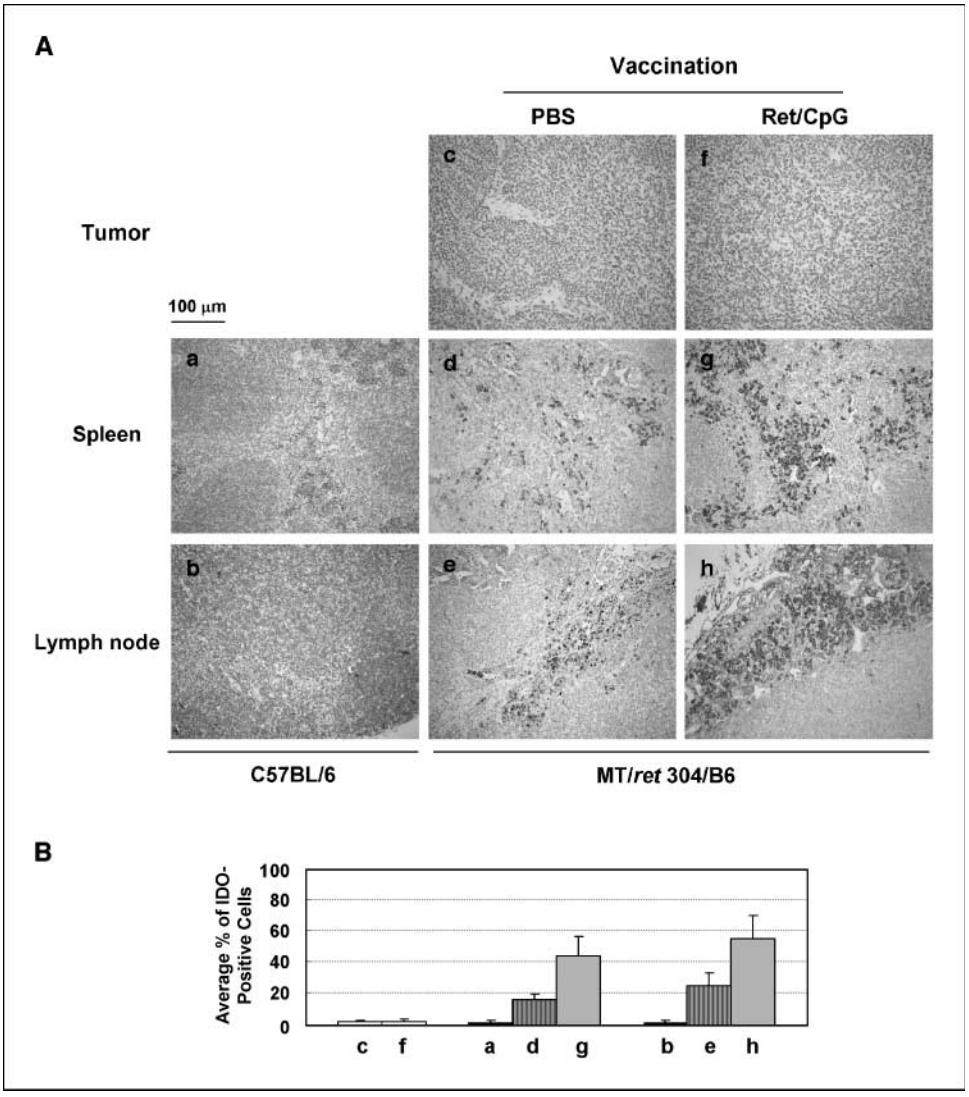

Figure 4. IDO expression in tissues. Tumor tissues, spleen, and lymph nodes from cervical sites were removed from mice and immunohistochemical IDO^+ assays were done. Representative examples from at least five identical experiments (×200). *A,* immunohistochemical analysis of IDO expression of different tissues. *a* and *b,* spleen and lymph nodes of C57BJ/6 mice. *c* to *e*, tumor, spleen, and lymph nodes of nonimmunized MT/*ret* 304/B6 mice. *f* to *h*, tumor, spleen, and lymph nodes of immunized MT/*ret* 304/B6 mice. *B,* measurement of the staining intensity of IDO expression of the tissues by National Center for Biotechnology Information ImageJ. *a* to *h,* samples as marked in *A*, respectively. Histogram shows the average percentage of IDO expression [% = brown area / brown (IDO staining) + blue (nuclear staining) × 100].

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immunity. According to the data from this study, the mice immunized with Ret vaccine generated a high level of specific anti-Ret antibody as well as exhibited highly specific activities of the CTL responses. These results suggested that the segment of Ret we selected had potent immunogenicity, which would be regarded as an ideal immunotherapeutic target for the associated carcinomas.

The IDO pathway has been cited as one important reason for immunologic tolerance to tumors (13–18). In most tumor-bearing hosts, a tolerant microenvironment is constructed through expression of IDO in tumors and host immune cells or only in immune cells to reject the host immune system. Lee and colleagues (14) found that IDO-positive cells were frequently host immune cells but not metastatic tumor cells. Munn and colleagues (15) reported that the IDO-positive cells in TDLNs seemed the host dendritic cells, and the IDO-positive dendritic cells proved to efficiently suppress the proliferation and the function of the activated T cells (17, 18). Using the spontaneous tumorigenic mouse model, we also observed that IDO was expressed in TDLNs and spleen but never tumor tissues in MT/*ret* 304/B6 mice immunized with or without the Ret/CpG vaccine. These findings implied that IDO-positive host antigen-presenting cells in TDLNs might be involved in the establishment of tumor immune tolerance, and the inhibition of IDO activity in immune cells would help improve the efficacy of cancer vaccines.

From the immunohistochemical results of IDO expression, we raise a question: Why is IDO expression strongly induced in the TDLNs and spleens of MT/*ret* 304/B6 mice immunized with the Ret/CpG vaccine? There are several possible ways to induce the expression of IDO. Among them, $CD4^+$ $CD25^+$ regulatory T cells have been clearly identified as a key component in inducing IDO expression, which serves as the generation of immunologic tolerance (34). Recent studies based on TCR transgenic mice displayed that regulatory T cells possessed self-antigen specificity because of their higher affinities to self-antigen (36, 37). Once activated, these regulatory T cells could be a factor that limited the activity of effector T cells and triggered high-level functional IDO expression in immune tissues, then resulting in immune tolerance. However, what is the precise mechanism of up-regulation of IDO expression in TDLNs and spleen of Ret/CpG-treated MT/*ret* 304/B6 mice and whether regulatory T cells are involved in this process remain to be elucidated.

We also noted that, in wild-type C57BL/6 mice, IDO expression was not detected in lymph nodes and spleen, although both humoral immunity and cellular immunity were strongly elicited due to the administration of the Ret/CpG vaccine. Correspondingly, MT/*ret* 304/B6 mice appeared to raise a high level of IDO expression in lymph nodes and spleen and displayed a strong specific humoral immune response with the Ret/CpG vaccination, but the tumor-specific CTL responses were not observed. Additionally, the inhibition of IDO activity by its specific inhibitor, 1MT, was efficient for repairing antigen-specific T-cell activation. Thus, it seems that IDO is presumed to mainly inhibit the Th1-polarized immune response in the vaccination progress and that the inhibition of IDO activity, as found previously in other studies (24–27), may be an available complementary approach in improving antitumor immunotherapy.

Additionally, in this study, we observed that administration of IFN-γ, a strong inducer for IDO expression, did not diminish (or even improved) the antitumor activity of Ret/CpG vaccine. The reasons

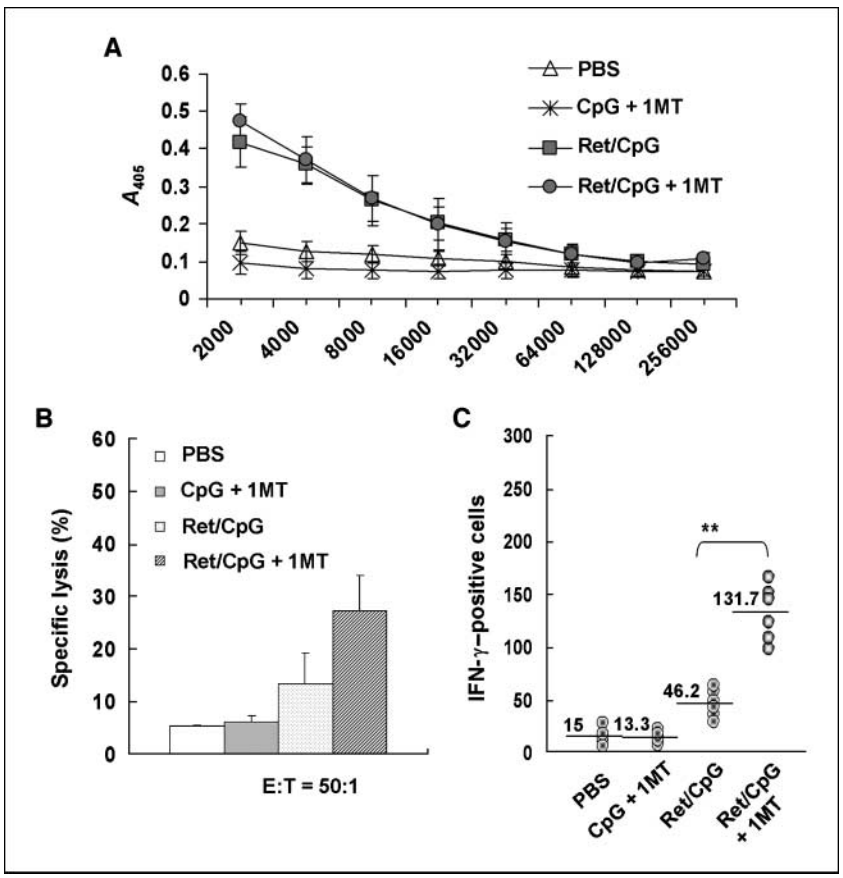

Figure 5. Effects of Ret/CpG vaccinations plus 1MT on immune responses in MT/*ret* 304/B6 mice. Mice were administered vaccinations plus DL-1MT gavage (400 mg/kg/dose, twice per day) as described in Materials and Methods (each data set in *A-C*; $n = 6$). *A,* ELISA results of serum anti-Ret antibody. *B,* tumor-specific CTL response analysis. *C,* Ret-specific IFN-γ ELISPOT assay analysis in splenocytes. **, $P < 0.01$.

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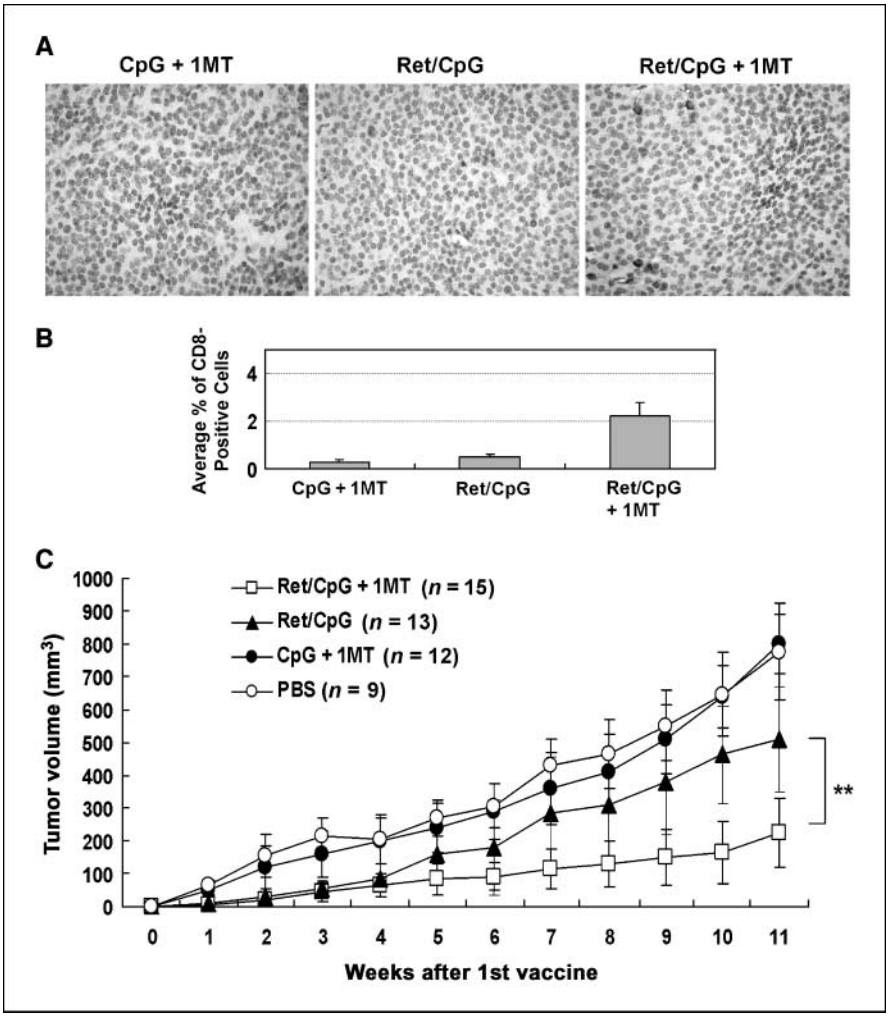

Figure 6. Effects of Ret/CpG vaccinations plus 1MT on delaying tumor growth in MT/*ret* 304/B6 mice. *A,* immunohistochemical $CD8^+$ assays in tumor tissue (×400). *B,* measurement of the staining intensity of CD8 expression by National Center for Biotechnology Information ImageJ. Average percentage of CD8 expression [% = brown area / brown (CD8 staining) + blue (nuclear staining) × 100]. *C,* weekly tumor growth analysis. **, $P < 0.01$ (n = 9-15).

involved this unexpected finding are probably to be complex and multifactorial. One explanation is that IDO does not promote tumor growth; therefore, its induction by IFN-γ does not weaken the efficacy of the Ret/CpG vaccine. In fact, it has been shown that up-regulation of IDO is able to stop the proliferation of malignant cells (38–41). Moreover, it is possible that 1MT has IDO-independent effects on malignant cells, which potentiate the activity of the vaccine. Accordingly, further studies are needed to clearly explain the relationship of blockage of IDO activity and cancer therapy.

In summary, our results suggest that the Ret/CpG vaccine plus 1MT management has a potent capacity to eliminate immune tolerance to tumors and that it has a potential preventive role in the immunotherapy of *ret*-associated carcinomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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