Synergistic *In vivo* Antitumor Effect of the Histone Deacetylase Inhibitor MS-275 in Combination with Interleukin 2 in a Murine Model of Renal Cell Carcinoma

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Abstract

Purpose: High-dose interleukin 2 (IL-2) is a Food and Drug Administration – approved regimen for patients with metastatic renal cell carcinoma. However, the toxicity and limited clinical benefit associated with IL-2 has hampered its use. Histone deacetylase (HDAC) inhibitors have been shown to have antitumor activity in different tumor models including renal cell carcinoma, and to have immunomodulatory properties. In our study, we tested the effectiveness of combination therapy of IL-2 with the HDAC inhibitor MS-275 in a murine renal cell carcinoma (RENCA) model.

Experimental Design: RENCA luciferase – expressing cells were implanted in the left kidney of BALB/C mice. Animals were randomly divided into four groups and treated with either vehicle, 150,000 IU of IL-2 twice daily by i.p. injections (twice weekly), 5 mg/kg of MS-275 daily by oral gavage (5 d/wk), or its combination. Treatment was started either 3 or 9 days following tumor cell injection.

Results: Weekly luciferase images and tumor weight after 2 weeks of treatment showed significant tumor inhibition (>80%) in the combination treatment as compared with the IL-2 (no significant inhibition) or MS-275 (~40% inhibition) treatment groups. Spontaneous lung metastases were also inhibited in the combination treatment (>90% inhibition) as compared with the single treatment group. Kaplan-Meier analyses showed statistically significant increased survival in the combination group as compared with controls and single agents. Splenocytes from mice treated with combination treatment showed greater lysis of RENCA cells than splenocytes from mice treated with single agents. The percentage of $CD4^+CD25^+$ T cells and $Foxp3^+$ T cells (T regulatory cells) was increased or reduced, respectively, in lymph nodes from tumor-bearing animals treated with the combination of MS-275 and IL-2 as compared with control and single agents. Depletion of $CD8^+$ T cells abrogated the survival benefit from MS-275 + IL-2 combination.

Conclusions: These results show that the combination of IL-2 and MS-275 has a synergistic antitumor effect *in vivo* in an immunocompetent murine model of renal cell carcinoma. The antitumor effect was associated with the decreased number of T regulatory cells and the increased antitumor cytotoxicity by splenocytes. In conclusion, these preclinical data provide the rationale for clinical testing of the combination of IL-2 and HDAC inhibitors in the treatment of patients with renal cell carcinoma.

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More than 51,000 new cases of carcinoma of the kidney and renal pelvis, and ~13,000 cancer-specific deaths in the U.S. have been estimated for 2007 (1). One third of patients have metastatic disease at the time of diagnosis. The prognosis for recurrent or metastatic renal cell carcinoma is poor, as median survival is 10 to 13 months, and 5-year survival is <5%. These figures underscore the need for effective systemic therapy for this disease.

In 1992, high-dose bolus interleukin 2 (IL-2) was approved by the Food and Drug Administration for the treatment of patients with metastatic renal cell cancer based on data presented on 255 patients who entered phase II clinical trials (2–4). High-dose bolus IL-2 has been shown to be superior

in terms of response rate and response quality to regimens involving either intermediate or low-dose IL-2, or s.c. IFNα (5). The response rate for high-dose IL-2 was 23% (22 of 95) versus 10% (9 of 91) for IL-2 plus IFNα. The median response durations were 24 months for high-dose IL-2 and 15 months for IL-2/IFN (P = 0.18). Another three-arm randomized study compared response rates and overall survival for patients with metastatic RCC receiving high-dose or one of two low-dose IL-2 regimens (6). Major tumor regressions, as well as complete responses, were seen with all regimens tested, but high-dose IL-2 was more clinically active, although this did not produce an overall survival benefit. Taken together, these data suggest that high-dose IL-2 should remain the preferred therapy for appropriately selected patients with access to such therapy before or after the use of the receptor tyrosine kinase inhibitors sorafenib and sunitinib. These two multikinase inhibitors have been recently approved by the Food and Drug Administration for the treatment of advanced kidney cancer (7, 8). Given the limited efficacy of high-dose IL-2 therapy, additional efforts should be directed to increase the efficacy of immunotherapy.

Histone deacetylases (HDAC) are critically important in gene and protein expression regulation, and represent a rational target for therapeutic interventions. The inhibitors of HDAC present a novel approach to the treatment of solid tumors. HDAC inhibitors induce cell cycle arrest, differentiation or apoptosis *in vitro*, and have potent antitumor activities *in vivo* (9–12). The synthetic benzamide MS-275 has shown induction of chromatin hyperacetylation and antitumor activity by inhibition of HDAC enzyme activity (13). MS-275 has also shown inhibition of tumor cell growth in nude mice that was comparable or superior to conventional cytotoxic agents. Our group has previously reported that MS-275 has a significant antitumor activity in a renal cell carcinoma model (14). The results from a phase I testing of MS-275 have also been recently reported (15).

Major histocompatibility class II proteins present antigenic peptides to T cells and are critical for the specificity and efficiency of the immune response. Major histocompatibility class II products may also contribute to the recognition of tumor cells by $CD4^+$ T cells and antitumor immunity (16). Several reports have established the importance of histone acetylation as a positive regulator of major histocompatibility class II transcription (17). A recent study has shown that the entire major histocompatibility class II family and the adjacent histone cluster located in the chromosome 6p21-22 locus are strongly induced by trichostatin A (18). HDAC inhibitors also have immunomodulatory properties including activation of costimulatory molecules CD40, CD80, and CD86 (19, 20). An emerging view is that helper T cells also use epigenetic mechanisms tied to the structure of chromatin and its covalent modifications to achieve important features of their programmed gene expression (21). Taken together, these data, along with the results from the clinical trials with HDAC inhibitors in patients with cutaneous T cell lymphoma and large cell lymphoma, suggest that the antitumor activity of HDAC inhibitors may be in part due to the modulation of the immune response.

In this preclinical study, we tested the hypothesis that combination therapy with IL-2 and the HDAC inhibitor MS-275 may have a greater antitumor effect, as compared with single agents, in an orthotopic murine renal cell carcinoma (RENCA) model.

Materials and Methods

Cell lines and reagents. The murine renal cell carcinoma cell line RENCA was purchased from American Type Culture Collection. Luciferase expression gene was transfected with lentivirus vector and RENCA was cultured in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (Sigma-Aldrich) and 1% Pen/Strep (Life Technologies), respectively, and incubated at 37°C in an atmosphere containing 5% CO_2. For the *in vitro* experiments, tumor cells were treated with IL-2 (Chiron), MS-275 (kindly provided by Schering AG, Berlin) or with a vehicle (DMSO). For the *in vivo* experiments, IL-2 was suspended using HBSS (Life Technologies), whereas the MS-275 was suspended in DMSO (Sigma-Aldrich).

***RENCA proliferation* in vitro.** RENCA tumor cell proliferation was assessed in an XTT assay according to standard procedures (Roche). RENCA cells were plated on day -1 at 1,000 cells per well in a 96-well plate in 10% RPMI. At day 0, cells were washed extensively and starved without serum. On day 1, cells were incubated in RPMI (5% serum) with increasing concentrations of MS-275 and/or IL-2. IL-2 and MS-275 concentrations for *in vitro* studies were chosen based on published reports (13, 14, 22). XTT assay was done on day 1 (as T = 0) reading and on day 4 (T = 72 h) according to the manufacturer's standard guidelines.

***Tumor growth* in vivo.** The animal protocol was approved by the Institutional Care and Use Committee at the Johns Hopkins Medical Institution, and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female 4- to 6-week-old BALB/c or male athymic nude mice (National Cancer Institute) were kept in a temperature-controlled room on a 12/12 h light/dark schedule with food and water *ad libitum*. Cells (5×10^4) harvested from nonconfluent monolayer cell cultures in 50 μL of medium were injected under the renal capsule without opening the peritoneum. The skin incision was closed with autosuture clips. Luciferase expression was determined 2 and 8 days after kidney injection in the prevention and intervention models, respectively. Based on luciferase expression levels, 25 to 40 animals were placed in four homogenous groups (5-10 animals/group): control, IL-2, MS-275, and combination. Animals in the control group were treated with a daily administration (5 d/wk) of the vehicle (DMSO) by gavage. Mice were given 150,000 IU of IL-2, twice a day, 2 days per week i.p., and/or with either 5 or 20 mg/kg of MS-275, 5 days a week by oral gavage. IL-2 and MS-275 doses for *in vivo* studies were chosen based on published reports (13, 14, 23). Animals were injected with luciferin i.p and luciferase expression of the tumor was measured by bioluminescence technology (Xenogen IVIS 100). Measurements were done once a week and reported as mean fluorescence intensity (SFI) ± SE. The animals were treated for 2 weeks and then euthanized by carbon dioxide inhalation. In the survival studies, the end point was the percentage of animals alive at different time points. Moribund animals were euthanized. At the end of the experiment, both primary tumors and lungs were collected. The weight of the healthy right kidney was subtracted from the RENCA-injected kidney. The number of metastases in three lung cross-sections per specimen was then evaluated.

Isolation and analyses of lymphocytes. For analysis of natural killer, natural killer T cells, $CD4^+$, and $CD8^+$ T cells, lymph nodes from three mice were processed per group and pooled. Each lymph node was smashed on slides and passed through a 100 μm nylon mesh filter into a 50 mL conical and brought to a volume of 45 to 50 mL. This suspension was spun at 1,500 rpm for 10 min at 4°C. The supernatant was aspirated and cell pellets resuspended in 10 mL of ACK Lysing Buffer (Biosource), incubated for 6 min on ice and centrifuged at 1,500 rpm for 10 min at 4°C. Supernatants were aspirated and pellets were resuspended in 5 mL of conditioned media.

Cell staining and flow cytometry. Following the isolation of lymphocytes from the lymph nodes of three mice, the cells were washed with flow buffer which included PBS with 1% of fetal bovine serum and 2 mmol/L of EDTA, then stained with CD3-FITC, CD3-PE,

CD4-FITC, CD8-cychrome, DX5-PE, CD25-APC (BD Pharmingen) and assayed on a FACScalibur flow cytometer (BD Biosciences).

Intracellular cell staining. After staining with anti-CD4 and CD25 antibodies following the above protocol, cells were washed in cold flow buffer, resuspended in 1 mL of cold Fix/Perm buffer (eBioscience), and incubated at 4°C overnight in the dark. After washing with flow buffer, cells were treated twice with permeabilization buffer (eBioscience). Cells were then blocked with Fcγ III/II R Ab (BD Pharmingen) for 20 min, and incubated with anti-mouse Foxp3 (FJK-16) antibody (eBioscience) for 45 min. Thus, cells were washed with permeabilization buffer twice, resuspended with flow buffer, and assayed on a FACScalibur flow cytometer (BD Biosciences).

Cytotoxic assay. Target cells (RENCA) were labeled with 100 mCi sodium chloride (Amersham Biosciences) per 3 to 5 million cells for 1 h. Target cells were seeded in 96-well V-bottomed plates at the indicated effector/target ratios against 1×10^4 effector cells (splenocytes). The release of ^{51}Cr from lysed target cells was counted on a gamma counter (Atlantic Nuclear) after 4 h of incubation at 37°C in 5% CO_2. The percentage of specific ^{51}Cr release was calculated by the following formula: percentage of specific lysis = (sample cpm - spontaneous cpm) × 100 / (maximum cpm - spontaneous cpm). Spontaneous cpm was calculated from the supernatant of the target cells alone, and the maximum release was obtained by adding 1N HCl to target cells.

In vivo ***depletion of CD8⁺ T cells.*** To deplete $CD8^+$ T cells, mice were injected with 250 μg of mouse monoclonal antibodies against $CD8^+$ T cells (2.43; Lofstrand Labs Limited) or HBSS only (control) on 8 days, 4 days, and 1 day prior to the tumor challenge. The anti-CD8 antibody administration was also repeated 6 days after tumor challenge, and then once a week. Flow cytometric analysis was done verifying 99% depletion of $CD8^+$ T cell subsets in the serum after the administration of depleting antibodies (data not shown).

Statistical analysis. Differences between the means of unpaired samples were evaluated by Student's *t* test using the SigmaPlot and SigmaStats program. $P < 0.05$ was considered statistically significant. All statistical tests were two-sided. Kaplan-Meier analyses were done for the survival studies and statistical significance was measured by using the log-rank test.

Results

The combination of MS-275 and IL-2 has a synergistic antitumor effect on RENCA primary tumor growth. We recently reported that MS-275 has inhibitory activity in a human renal cell carcinoma model (14). To determine whether MS-275 inhibits RENCA cell proliferation, we conducted *in vitro* experiments (Fig. 1). Increasing concentrations of IL-2 (0.005-0.15 million IU/mL) did not have any inhibitory effect on RENCA cell proliferation, whereas a dose-dependent inhibition was observed with MS-275 (0.25-2 μmol/L; Fig. 1A and B). The combination of MS-275 and IL-2 did not show a greater inhibitory effect as compared with single-agent MS-275 (Fig. 1C). To assess the effect of MS-275 and IL-2 on RENCA growth *in vivo*, luciferase-expressing RENCA cells were injected orthotopically in immunocompetent mice. Animals received either control vehicle, IL-2 (150,000 IU twice a day, twice a week for 2 weeks), MS-275 (5 mg/kg/d), or the combination. Treatment of RENCA-bearing animals with single-agent IL-2 did not show a significant inhibitory effect as assessed by bioluminescence technology in a real-time fashion. MS-275 administration induced a 40% reduction of tumor growth. However, the combination of MS-275 with IL-2 had a synergistic inhibitory effect compared with single agents alone (90% reduction in luciferase expression). Differences in luciferase expression correlated with tumor weights (Fig. 2A).

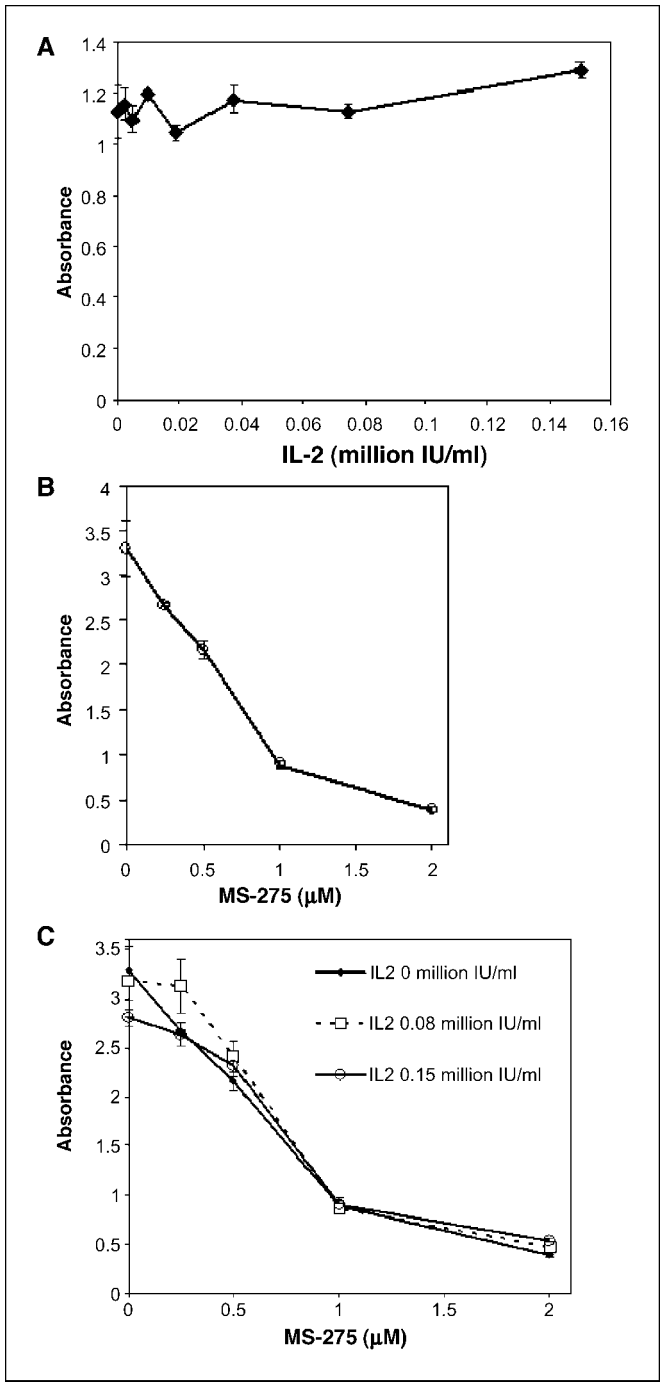

Fig. 1. Antitumor effect of IL-2 and MS-275 *in vitro*. RENCA cells were treated with either increasing doses of (*A*) IL-2 (0.005-0.15 million IU/mL), (*B*) MS-275 (0.25-2 μmol/L), or (*C*) its combination. Points, mean absorbance; bars, SE. The experiment was repeated twice with similar results.

Histologic evaluation of tumor samples showed that the renal parenchyma was completely replaced by tumor cells in controls and IL-2–treated groups. In the MS-275–treated group, normal renal parenchyma was identifiable in the context of tumor infiltration. No microscopic tumor cell infiltration was observed in the combination group except for one sample (Fig. 2B, see *arrows*). The antitumor effect of this combination was evaluated by luciferase expression both in a "prevention" and

an "intervention" model, in which treatment was started 3 and 9 days following RENCA implantation, respectively. In the prevention model, the combination treatment had profound inhibitory effects on tumor growth ($P < 0.05$; Fig. 2C). Tumor weight measurements following 2-week treatment revealed significant inhibition of tumor growth as a result of the combination treatment, whereas MS-275 yielded a greater inhibitory effect than IL-2 alone or control ($P < 0.05$; Fig. 2D).

Combination of MS-275 and IL-2 inhibits spontaneous RENCA lung metastases. RENCA tumor cells implanted in the renal capsule metastasize spontaneously to regional lymph nodes and lungs. Thus, we examined the lungs of mice treated with IL-2, MS-275, or its combination. We observed the presence of macroscopic lung metastases in all control and IL-2–treated animals. Lungs collected from MS-275–treated mice revealed pulmonary nodules in five out of seven animals. However, combination treatment with MS-275 and IL-2 had a significant inhibitory effect and only one out of seven animals revealed the presence of macroscopic lung metastases (Fig. 3A). Lungs were also assessed by histologic examination. The results confirmed that although MS-275 treatment had an antimetastatic effect, the combination treatment with IL-2 had a greater effect as compared with MS-275 alone (Fig. 3B). Histologic evaluation of the lung sections revealed that the group treated with MS-275 showed a smaller number of metastases as compared with control and IL-2–treated groups. However, only one mouse in the group treated by combination treatment showed the presence of lung RENCA colonies (Fig. 3C).

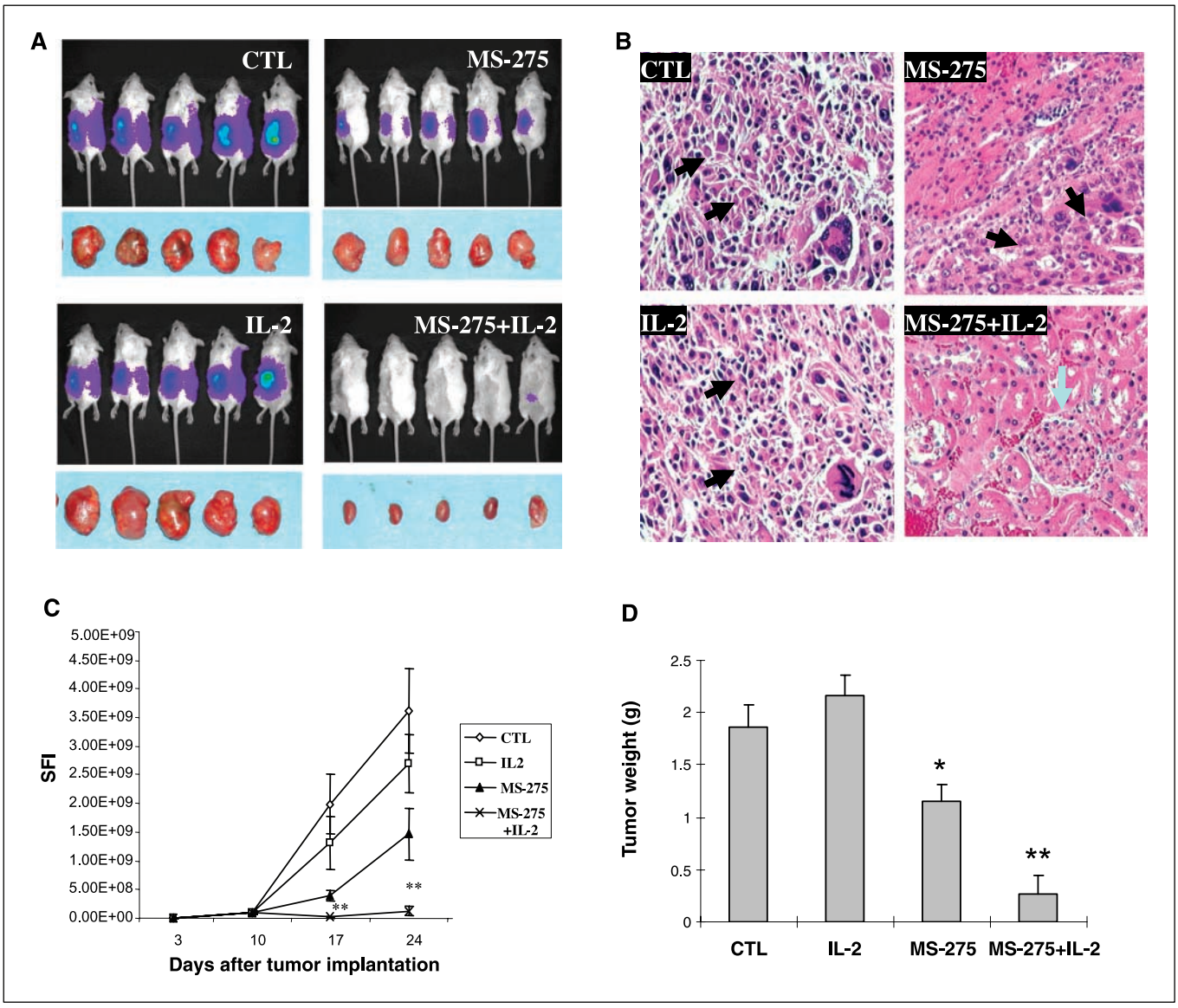

Fig. 2. Antitumor effect of IL-2 and MS-275 *in vivo. A,* luciferase expression and macroscopic RENCA tumors following treatment with MS-275 (5 mg/kg/d), IL-2 (1.5×10^5 units twice a day, twice a week) or its combination, in the prevention model. *B,* H&E staining of RENCA tumors (magnification, ×100). Presence of tumor cells (*black arrows*); a glomerulus in the context of normal renal tissue in the combination group (*white arrow*). *C,* real-time quantitative analysis of luciferase expression in the prevention model (treatment started 3 d post-tumor implantation). Points, mean fluorescence intensity (SFI); bars, SE (*, $P < 0.05$ versus control; **, $P < 0.05$ versus single agents). *D,* tumor weight measurements. Columns, mean grams of tumor; bars, SE (*, $P < 0.05$ versus control; **, $P < 0.05$ versus single agents). The experiment was repeated thrice with similar results.

Combination of IL-2 and MS-275 prolongs survival. Thus, we assessed whether the significant antitumor effect achieved by this combination in the RENCA model also translated in a survival advantage. As shown in Fig. 4A, the combination treatment in the prevention model resulted in a statistically significant improvement in the survival of RENCA tumor–bearing animals as compared with either MS-275 ($n = 9$, $P < 0.0001$), IL-2, or control ($n = 9$, $P < 0.0001$) groups. The survival curve in the intervention model also showed that the combination treatment prolonged survival in mice with established tumors as compared with either MS-275, IL-2, or control ($n = 9$, $P < 0.05$; Fig. 4B) groups.

Antitumor effects of IL-2 and MS-275 in immunodeficient murine model **in vivo.** The antitumor effects of IL-2 and MS-275 were also evaluated in immunodeficient mice following luciferase expression. Treatment was started 3 days after RENCA cell implantation. There was no significant difference in luciferase expression by using IL-2, MS-275, or its combination (Fig. 5A). Tumor weight measurements 3 weeks after tumor cell injection revealed a nonstatistical significant inhibition of MS-275 and combination on RENCA growth (Fig. 5B). No difference in tumor volume and weight between MS-275 and combination groups was observed in RENCA-bearing nude mice.

MS-275 treatment affects $CD4^+$ $CD25^+$ T cells in lymph nodes. Thus, we decided to explore the potential mechanisms underlying the antitumor effect elicited by the combination of IL-2/MS-275. In view of the lack of synergistic effect of MS-275 and IL-2 in immunodeficient athymic mice, we hypothesized that a T cell–mediated mechanism may in part be responsible for the observed biological phenomenon. In separate experiments, we assessed specific subsets of lymphocytes in loco-regional lymph nodes from tumor-bearing BALB/c mice. Loco-regional lymph nodes were collected on day 14 after the initiation of treatment in the RENCA prevention model. Lymphocytes were isolated and analyzed by fluorescence-activated cell sorting. As shown in Fig. 6A, the total $CD4^+$ T cell population in the untreated controls was lower than in the treatment groups. Interestingly, the highest level of $CD4^+$ T cells was observed in the combination-treated animals. The population of regulatory T cells ($CD4^+$ $Foxp3^+$) was decreased in the MS-275 group and, to a greater extent, in the combination-treated

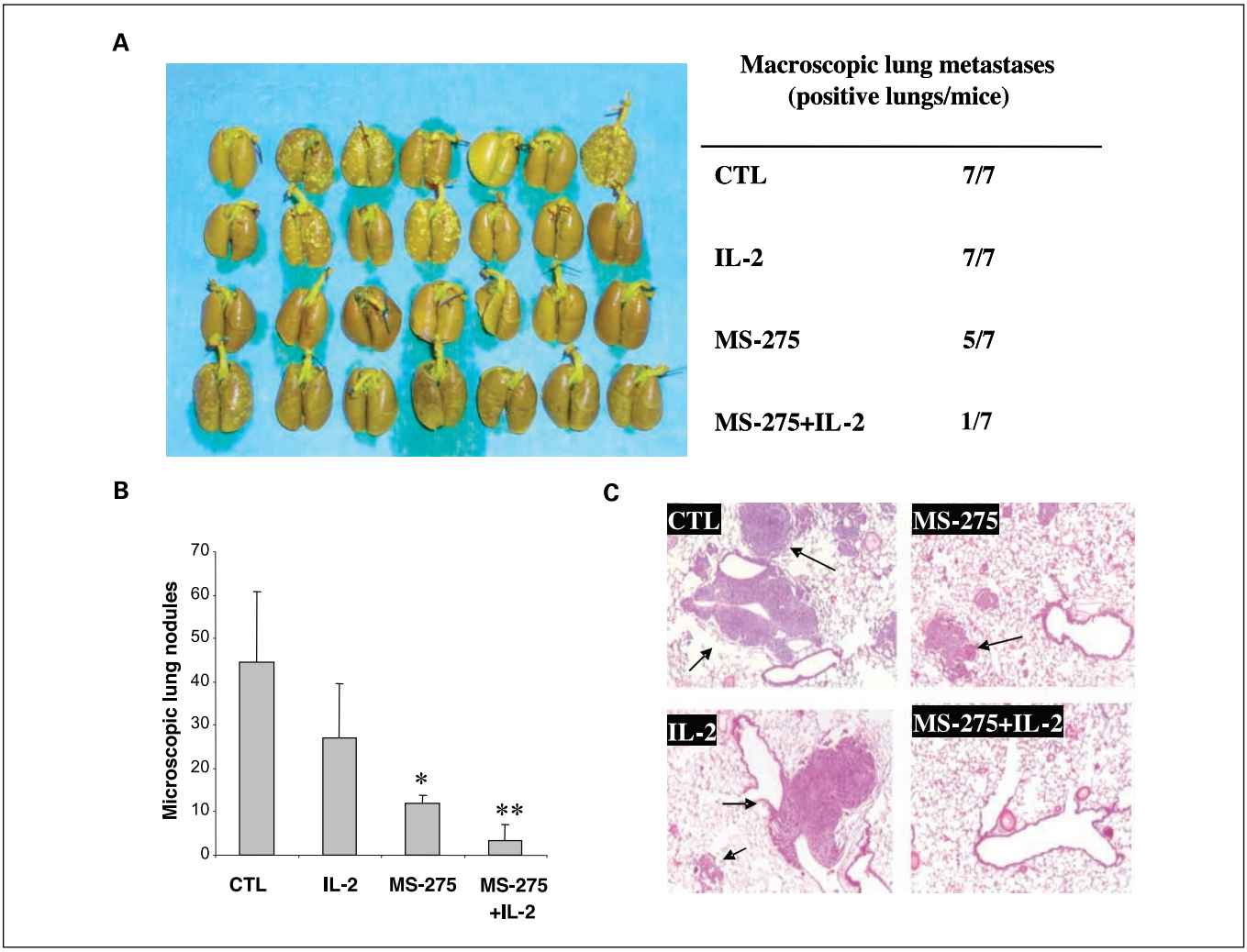

Fig. 3. Combination of IL-2 and MS-275 prevents lung metastases. *A,* macroscopic spontaneous lung metastases and incidence in RENCA tumor – bearing animals treated with IL-2, MS-275, or its combination. *B,* quantitative analysis of microscopic lung metastases. Columns, mean lung nodules; bars, SE (*, $P < 0.05$ versus control; **, $P < 0.05$ versus single agents). *C,* H&E staining of lung specimens (magnification, ×100). Lung metastases (*arrows*). The experiment was repeated three times with similar results.

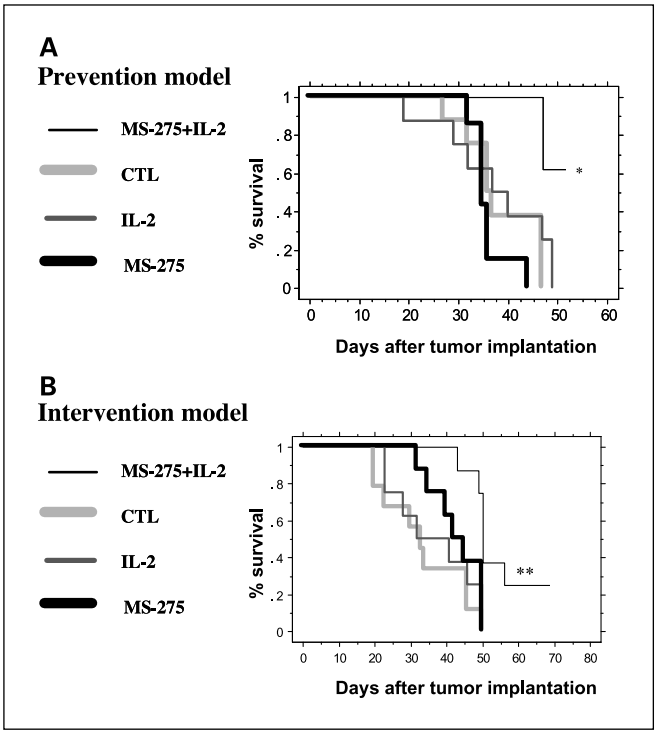

Fig. 4. Combination of IL-2 and MS-275 prolongs survival in RENCA tumor–bearing animals. Kaplan-Meier survival curves from the treatment groups in the prevention (*A*) and intervention (*B*) models. Statistical significance was measured by using log-rank test. $n = 9$, $P < 0.0001$ versus MS-275 (*A*); $n = 9$, $P < 0.05$ versus MS-275 (*B*). The experiment was repeated twice with similar results.

animals (Fig. 6A). These experiments were repeated thrice and the average values are shown. No significant differences were observed in the levels of $CD8^+$, natural killer, and natural killer T cells among the different groups (data not shown).

In vivo *combination of MS-275 and IL-2 induces cytotoxic T cells.* We assessed whether the combination of IL-2 with MS-275 was associated with an increased immune cell–mediated direct cytotoxic effect. BALB/c mice were treated with MS-275, IL-2, or its combination for 5 days. Then, spleens were collected and isolated splenocytes were tested in a cytotoxic *in vitro* assay. Splenocytes were used as effectors cells whereas RENCA cells were the target cells. As shown in Fig. 6B, splenocytes isolated from the combination group induced lysis of RENCA cells. Splenocytes isolated from IL-2–treated mice had a lower cytotoxic effect. No cytotoxic activity was observed with splenocytes isolated from either control or MS-275–treated animals. Under our experimental conditions, the overall low levels in cytotoxic activity of splenocytes, even in the combination group, may be explained by the absence of *in vitro* IL-2 stimulation which is routinely done in this assay. To explore the potential mechanisms responsible for the *in vivo* MS-275 + IL-2–induced cytotoxic effect, we induced $CD8^+$ T cell depletion in tumor-bearing animals. Survival benefit from MS-275 + IL-2 treatment was abrogated in animals treated with the anti-CD8 antibody (Fig. 7A). Death was associated with large tumors (Fig. 7B).

Discussion

We have previously reported that the HDAC inhibitor MS-275 has antitumor activity in a human renal cell carcinoma model (14). In the current study, we tested the hypothesis whether MS-275 treatment enhances the antitumor activity of IL-2. Our results revealed that the combination of MS-275 and IL-2 has a significant antitumor effect on primary tumor growth in an orthotopic murine renal cell carcinoma model as compared with single agents.

To explore the potential mechanisms responsible for the synergistic effect of this combination, we assessed the levels of $CD4^+$ T cells from regional lymph nodes in tumor-bearing animals by using fluorescence-activated cell sorting analysis. Combination treatment with MS-275 and IL-2 increased the ratio of $CD4^+$ T cells/total lymphocytes in the abdominal lymph nodes as compared with control and single agents. Interestingly, when we depleted the RENCA-bearing mice of $CD8^+$ T cells, the survival benefit from MS-275 + IL-2 treatment was dramatically reduced. This is in agreement with previous reports showing $CD8^+$ T cells as critical components for cytokine-based immunotherapies in the RENCA model (24). Taken together, these results suggest that the synergistic activity of this strategy may be due to the opposite action of MS-275 and IL-2. IL-2 treatment may increase the number of effector T cells that are decreased by MS-275, whereas MS-275 treatment may inhibit T regulatory cells (T_{regs}) which are induced by IL-2. Thus, IL-2 treatment in combination with the HDAC inhibitor MS-275 may restore the number of effector T cells without inducing the regulatory T cells.

The observed potential inhibitory effect of MS-275 on T_{regs} is of particular interest in view of the immunosuppressive role of this T cell subset. T_{regs} represent a subset population

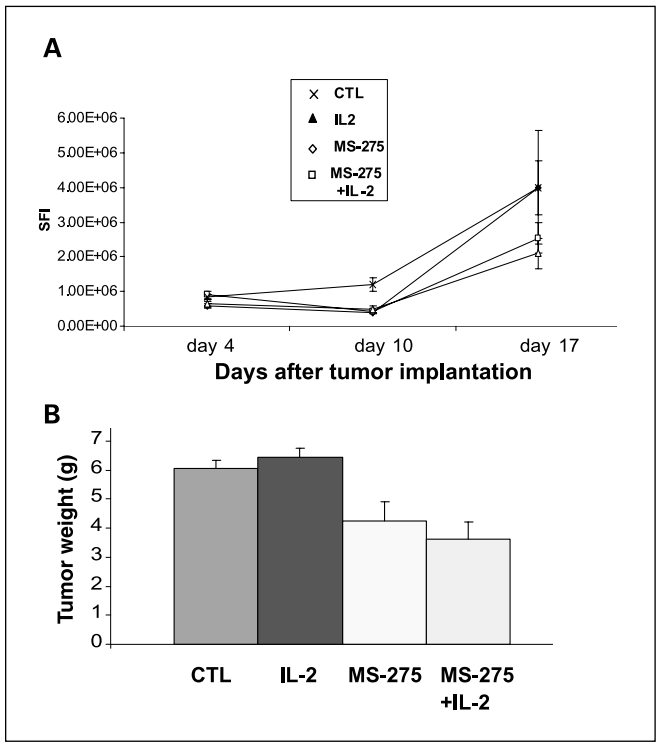

Fig. 5. Combination of IL-2 and MS-275 does not have a synergistic effect in immunodeficient mice. *A,* luciferase expression was evaluated in RENCA-bearing athymic nude mice in which treatment was started 3 d after RENCA injection. Points, mean fluorescence intensity; bars, SE. *B,* tumor weight measurements. Columns, mean grams of tumor; bars, SE. The experiment was repeated thrice with similar results.

of T cells ($CD4^+CD25^+Foxp3^+$) that have been associated with suppression of self-reactive T cells (25, 26). T_{regs} may also impair the function of effector T cells in cancer patients and induce immune tolerance (27). Activated T_{regs} show antigen-nonspecific suppressor activity *in vitro* (28). IL-2 plays a critical role in T cell clonal expansion, including T_{regs}, and enhances effector T cell development (29). In our study, the population of T_{regs} decreased in the combination treatment group as compared with either control or IL-2. The number of T_{regs} in the MS-275 and the combination treatment groups was similar, and it was ~50% as compared with either control or IL-2–treated groups. Two recent reports suggest that T_{regs} are induced in cancer patients receiving high-dose IL-2 (30, 31). Interestingly, high-dose IL-2 resulted in a significant decrease in T_{regs} in those patients achieving an objective clinical response to IL-2 therapy (30). The mechanism responsible for this finding remains unclear. These clinical reports suggest that depletion of regulatory T cells may enhance the ability of IL-2 to elicit antitumor immune response in cancer patients. Further studies will be necessary to confirm the potential role of MS-275 in regulating T_{regs} and to elucidate the underlying molecular mechanisms.

In our model, we used a suboptimal dose of MS-275 (5 mg/kg rather than 30-40 mg/kg). When we used a higher dose of MS-275 (20 mg/kg), we did not observe the synergistic effect (data not shown). This observation suggests that the synergistic effect may be obtained with a relatively low dose of HDAC inhibitor. The median plasma concentration 30 min following MS-275 (5 mg/kg) administration was 20.6 ± 5.01 ng/mL. This pharmacokinetic analysis was done based on previously published methodology (32). Nanomolar peak plasma concentrations of MS-275 were able to induce the observed immunomodulation in the RENCA model. It is possible that maximum tolerated doses of HDAC inhibitors may not be necessary to achieve an optimal clinical benefit in combination with IL-2, and indeed, it may be detrimental because of its potential negative effect on effector immune cells. In a separate experiment, we also tested whether there

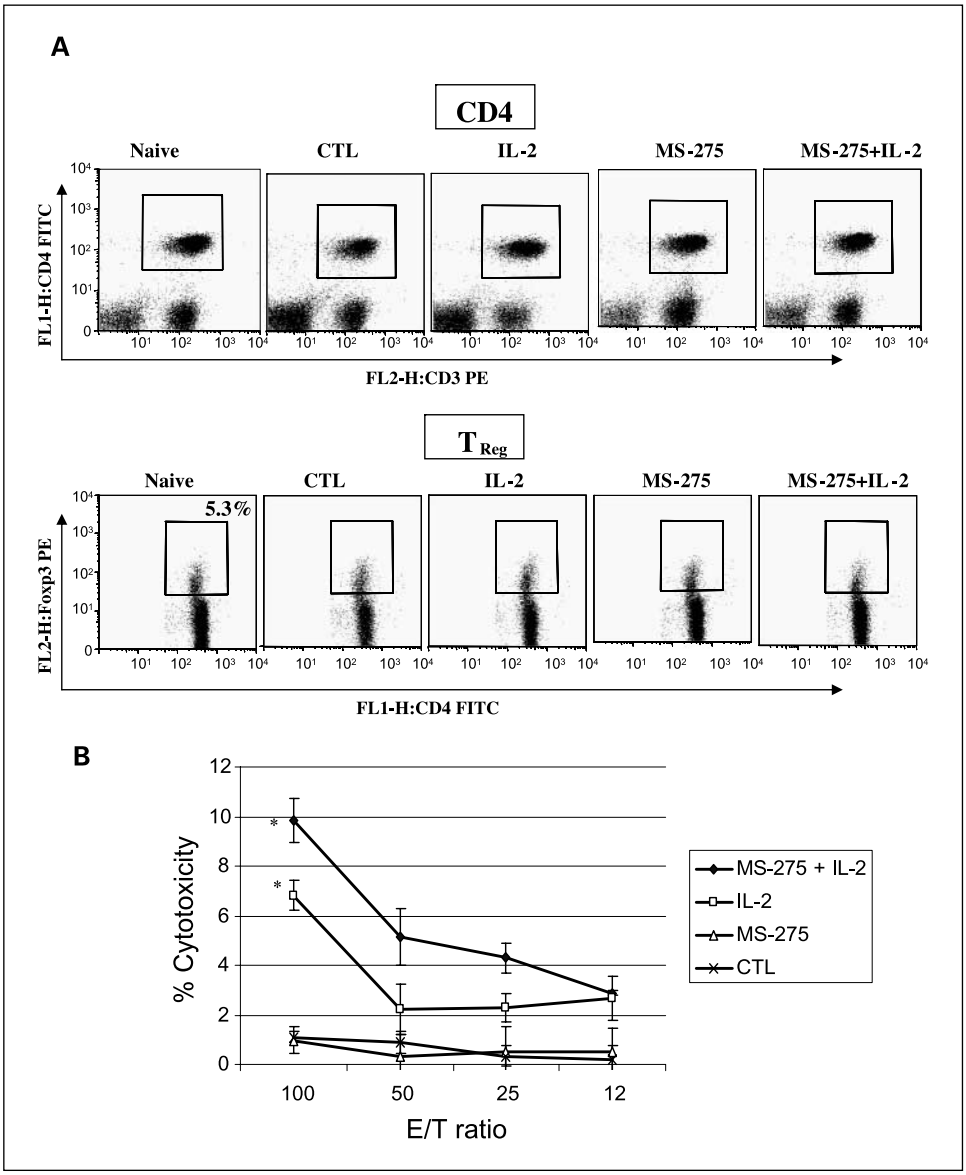

Fig. 6. Combination treatment with IL-2 and MS-275 was associated with a reduced T_{reg} percentage in regional lymph nodes and increased *in vitro* splenocyte cytotoxicity. *A,* population of $CD4^+$ and T_{regs} ($CD4^+$ $Foxp3^+$) lymphocytes from abdominal lymph nodes 8 d after kidney injection (5 d after treatment) was determined by fluorescence-activated cell sorting (percentage of positive cells). *B,* splenocyte-induced lysis of RENCA assessed by ^{51}Cr release assay. Two weeks after treatment, spleen cells were isolated from BALB/c mice and used as effector cells from five groups: untreated (*CTL*), MS-275 (5 mg/kg/d), IL-2 (1.5×10^5 units twice a day, twice a week), and combination. RENCA cell cells were used as target cells. The effector-target ratios were 100, 50, 25, and 12.5 in a volume of 200 μL for 4 h of incubation. Points, mean percentage of cytotoxicity; bars, SE (*, $P < 0.05$ versus control). The experiment was repeated three times with similar results.

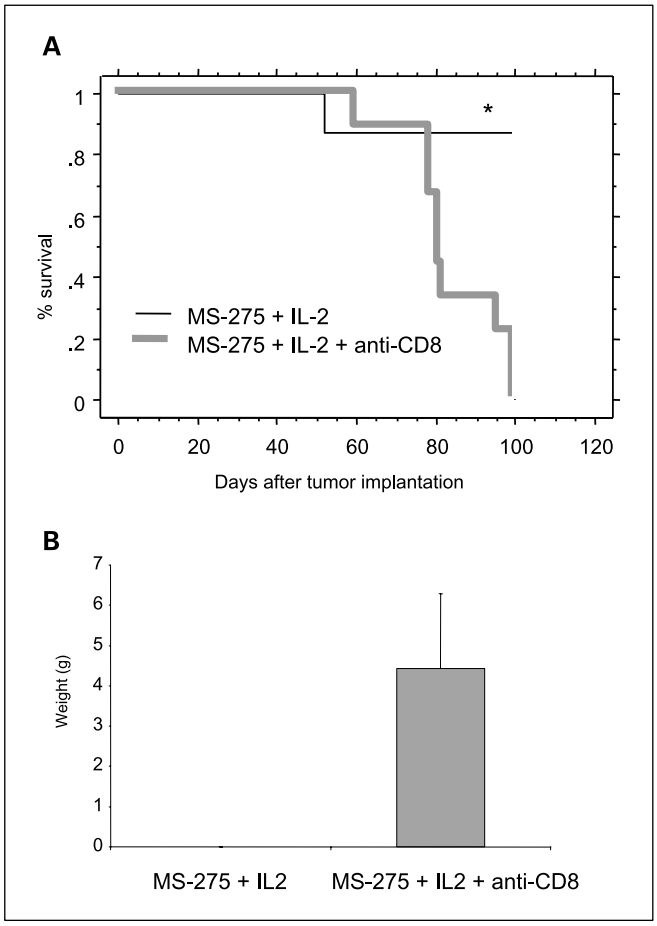

Fig. 7. Depletion of CD8 T cells is associated with reduced survival in mice treated with MS-275 + IL-2. *A,* Kaplan-Meier survival curves in the control and treatment groups in the prevention model. Statistical significance was measured by using log-rank test. $n = 9$, $P < 0.0001$ versus MS-275 + IL-2. *B,* tumor weight measurements. Columns, mean grams of tumor; bars, SE.

was a dose-dependent effect of IL-2. When we treated the RENCA-bearing animals with a lower dose of IL-2 (15,000 units) we still observed a greater antitumor effect of the combination treatment as compared with single agents (data not shown). However, the histologic examination revealed the presence of microscopic tumor in the majority of MS-275 + IL-2–treated mice. These results are consistent with a dose-dependent effect of IL-2 in this combination strategy and suggest that high-dose IL-2 should be preferred in the translation to the clinical setting.

Preclinical and clinical studies suggest that immunotherapy targeting specific tumor-associated antigens may be beneficial in patients with cancer (33, 34). Epigenetic modulation of gene expression by aberrant methylation of DNA in both tumor cells and lymphocytes has been reported to influence signaling and expression of proteins important for innate and acquired immune system (21, 35, 36). A report has shown that the DNA demethylating agent 5-aza-2′-deoxycytidine can induce *de novo* expression of a cancer/testis antigen in negative cancer cell lines and synergize with adoptive immunotherapy in a syngeneic murine mammary carcinoma model (37). More recently, Reu et al. have reported that treatment of human renal cell carcinoma and melanoma cells with 5-aza-2′-deoxycytidine synergistically augmented the antiproliferative effects of IFN (IFN-αand IFN-β; ref. 38). Either 5-aza-2′-deoxycytidine or an antisense to DNA methyltransferase 1 overcame resistance to apoptosis induction by IFN. Reactivation of the cancer-testis antigens MAGE and RAGE after DNA methyltransferase 1 depletion was also identified. Taken together, these data support the importance of epigenetic remodeling in immunomodifying gene expression and the potential role of inhibitors of DNA methyltransferase 1 in the augmentation of cytokine effects and/or expression of tumor-associated antigens. To date, there are no reports on HDAC inhibitors as modulators of tumor-associated antigens. Rational combinations of HDAC inhibitors, demethylating agents, and immunotherapy should be further explored in preclinical models to confirm the role of "epigenetic" therapy in enhancing the induction of immune response by modulating subsets of immune cells and inducing tumor cell antigen expression.

In conclusion, our preclinical results show, for the first time, that combination of IL-2 and an HDAC inhibitor, MS-275, has a synergistic antitumor effect *in vivo* in an immunocompetent murine model of renal cell carcinoma. The significant improvement of IL-2 therapy by the HDAC inhibitor MS-275 was associated with the decreased number of T_{regs} and increased cytotoxicity by splenocytes. Taken together, these data provide the rationale for a novel therapeutic regimen aimed to increase clinical response in patients with renal cell carcinoma. The immunomodulatory activity of MS-275 and its direct antitumor effect may increase the response rate to IL-2, further delaying disease progression and increasing progression-free survival in patients with metastatic renal cell carcinoma, and potentially, also in patients with other immunogenic tumors such as melanoma.

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