

# Macrophage-dependent nitric oxide expression regulates tumor cell detachment and metastasis after IL-2/anti-CD40 immunotherapy

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Using an orthotopic model of renal cell carcinoma, we showed previously that IL-2/anti-CD40 immunotherapy resulted in synergistic anti-tumor responses, whereas IL-2 or  $\alpha$ -CD40 alone mediated partial transient anti-tumor effects. We now show that treatment of tumor-bearing mice with IL-2/ $\alpha$ -CD40, but not IL-2 or  $\alpha$ -CD40, induced significant nitric oxide synthase (NOS) 2 expression in tumor-associated macrophages. In control-treated mice (low NO), NOS2 inhibition reduced tumor burden. However, during immunotherapy (high NO), NOS2 inhibition or macrophage depletion reversed the ability of IL-2/ $\alpha$ -CD40 treatment to reduce lung metastases but had no effect on primary tumor burden. Furthermore, IL-2/ $\alpha$ -CD40 induced the IFN- $\gamma$ - and NO-dependent decrease in matrix metalloproteinase (MMP) expression and activity, concomitant with increases in tissue inhibitor of metalloproteinase (TIMP) 1 and E-cadherin expression within tumors. Finally, treatment of tumor-bearing mice with the NO donor JS-K significantly reduced metastases. These data differentiate the mechanism for primary anti-tumor effects of IL-2/ $\alpha$ -CD40 immunotherapy, which are independent of NO, from the NO-dependent inhibition of metastases. Furthermore, reduced MMP9 activity implicates M1-polarized macrophages within the tumor microenvironment as critical components of therapeutic response. Our data demonstrate the mechanistic basis for IL-2/ $\alpha$ -CD40-mediated control of metastases and suggest that the context-dependent application of NO donors may hold promise for prevention of metastatic disease.

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Abbreviations used: GKO, IFN- $\gamma$  knockout; HPRT, hypoxanthine phosphoribosyltransferase; L-NAME, N $\omega$ -nitro-L-arginine methyl ester; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; NOS, NO synthase; RCC, renal cell carcinoma; TIMP, tissue inhibitor of metalloproteinase; VC, vehicle control.

The presence of a strongly immunosuppressive tumor microenvironment and metastasis at time of diagnosis represent two fundamental obstacles to the treatment of cancer. Tumor-associated macrophages are frequently observed to be immunosuppressive and functionally polarized to promote tumor growth and metastasis (Lin et al., 2001; Serafini et al., 2006a; Sica et al., 2008; Qian et al., 2009). Indeed, these studies have highlighted a direct correlation between extravasation and metastatic potential of tumors with the infiltration of tumors by macrophages (Lin et al., 2001; Qian et al., 2009). Although the complex interactions between macrophages and tumor cells are incompletely defined, it has become increasingly evident that the production of proteases, growth factors, and cytokines by

macrophages may enhance the efficiency of the metastatic process. However, macrophages exhibit a large degree of plasticity in that their cellular responses can be profoundly influenced by the cytokine and cellular environment. In this regard, IL-12 has emerged as a promising therapeutic agent in that it functionally alters tumor-associated macrophages toward an anti-tumor anti-metastatic profile (Watkins et al., 2007). Previously, we reported that IL-12-based combination cytokine immunotherapies, namely IL-2/IL-12 and IL-2/anti-CD40, can effectively alter the balance of the tumor microenvironment

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toward a beneficial host immune response (Wigginton et al., 1996a; Weiss et al., 2009). The IL-2/IL-12 immunotherapeutic regimen has demonstrated objective benefits in some patients with melanoma and renal cell carcinoma (RCC; Gollob et al., 2003).

Nitric oxide (NO) is a critical mediator of macrophage function, and its expression is classically associated with the cytotoxic activity of macrophages against transformed cells (Nathan and Hibbs, 1991; Farias-Eisner et al., 1994). Furthermore, IL-12 treatment primes macrophages *in vivo* for enhanced NO production, and macrophage-associated NO may be an important component of successful IL-12-based immunotherapies (Wigginton et al., 1996b). Within the tumor microenvironment, NO can be generated by macrophages, neutrophils, endothelial cells, fibroblasts, and, in certain cases, by the tumor cells themselves. Despite its established role in anti-tumor responses, NO has been hypothesized to have a dual role because under certain circumstances, NO expression promotes tumor progression (Orucevic et al., 1999). The reason for this apparent contradiction lies in the complex ability of NO to regulate diverse cellular processes, including cell adhesion, invasiveness and proliferation, matrix remodeling, and angiogenesis (for review see Williams and Djamgoz, 2005). Furthermore, NO synthase (NOS) 2 expression has been shown to contribute to some of the critical immunosuppressive properties of myeloid-derived suppressor cells (MDSCs) that are frequently associated with tumors (Serafini et al., 2006a), and NO inhibition can result in augmented anti-tumor responses through the reversal of MDSC-mediated suppression (Serafini et al., 2006b). The local concentration of NO may partially explain its biphasic nature in cancer. On the one hand, high steady-state concentrations of NO result in P53 phosphorylation, which is itself associated with tumor cell apoptosis, cell cycle delay, and DNA repair (Ambs et al., 1998; Thomas et al., 2004). High NO concentrations also impair the activity of matrix metalloproteinases (MMPs), which play important roles in matrix remodeling and the metastatic process (Liotta and Stetler-Stevenson, 1990; Ridnour et al., 2007). On the other hand, low concentrations of NO have been shown to promote the HIF-1 $\alpha$  and/or MAPK-mediated promotion of tumor growth (Thomas et al., 2004) and low NO concentrations actually enhance MMP activity by inhibiting their endogenous inhibitor, tissue inhibitor of metalloproteinase (TIMP) 1 (Ridnour et al., 2007). Collectively, the levels of NO achieved within the tumor microenvironment likely play a critical role in regulating the balance between tumor promoting and inhibiting properties of tumor-associated macrophages.

Previously, we showed that the IL-12-based immunotherapy IL-2/ $\alpha$ -CD40 elicits synergistic anti-tumor responses that are dependent on IL-12 (Murphy et al., 2003; Weiss et al., 2009). We hypothesize that this is, in part, dependent on the priming of macrophages to produce high levels of NO that help establish a polarized tumor microenvironment whereby the macrophages display a classical M1 phenotype, characterized not only by high levels of NO production but also by

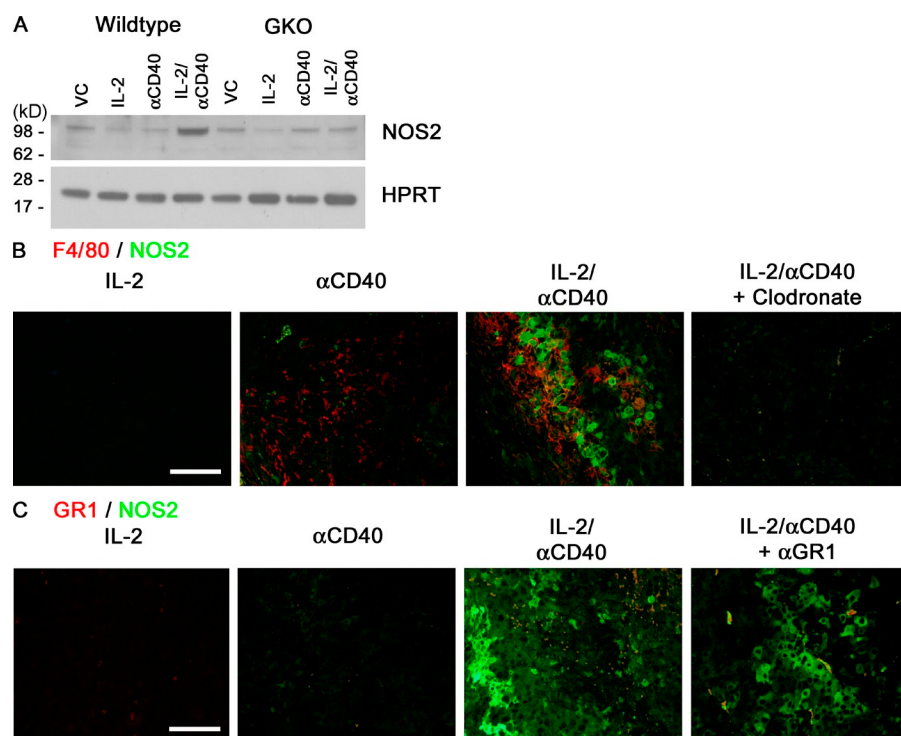
reduced arginase expression concomitant with the production of Th1 cytokines (e.g., IFN- $\gamma$ , MIG, IP-10, and RANTES) by other inflammatory leukocytes that are associated with favorable prognosis (Wigginton et al., 1996b; Weiss et al., 2009). In this study, we confirm and extend this hypothesis by demonstrating that the anti-metastatic potential of IL-2/ $\alpha$ -CD40 immunotherapy is critically dependent on macrophage-dependent NO expression within the tumor microenvironment. Furthermore, we demonstrate, for the first time, a clear example whereby a successful immunotherapeutic regimen against a primary tumor is NO independent, whereas concomitant control of metastatic spread to lungs is NO dependent. Importantly, IL-2/ $\alpha$ -CD40 induced the reduction in MMP expression and activity and increased E-cadherin expression. Thus, IL-2/ $\alpha$ -CD40 achieves a reduction in the ratio of MMP/E-cadherin expression within the tumor microenvironment that has been associated with favorable prognosis in patients with metastatic disease (Kuniyasu et al., 1999; Slaton et al., 2001; Fukata et al., 2005; Campos et al., 2006; Pettaway et al., 2008). Finally, we show that treatment of tumor-bearing mice with an NO donor can elicit anti-metastatic effects by directly acting on the primary tumor. These findings highlight the feasibility of using NO-inducing agents and combination immunotherapy to construct new strategies for therapy of metastatic disease.

## RESULTS

### IL-2/ $\alpha$ -CD40 induces NOS expression in tumor-associated macrophages

Previously, we showed that IL-2/ $\alpha$ -CD40 combination immunotherapy rebalances the profile of tumor-infiltrating leukocytes toward that of an anti-tumor Th1/M1-like phenotype (Weiss et al., 2009). Furthermore, we showed that IL-2/ $\alpha$ -CD40 induced a significant reduction in arginase expression in tumor-associated macrophages. The balance between arginase and NO expression by tumor-infiltrating macrophages has been associated with the ability of these cells to promote or inhibit tumor development, respectively (Sica et al., 2008). We therefore analyzed NOS2 expression in primary tumors after IL-2/ $\alpha$ -CD40 therapy.

By Western blot analysis of tumor lysate samples, we found that IL-2/ $\alpha$ -CD40 increased NOS2 expression, as compared with control, IL-2, or  $\alpha$ -CD40 treatment (Fig. 1 A). By performing the experiment in IFN- $\gamma$  knockout (GKO) mice, we confirmed that endogenous IFN- $\gamma$  was required for the IL-2/ $\alpha$ -CD40-mediated induction of NOS2 in tumors (Fig. 1 A). Next, we determined the cellular source of NOS2 expression in the tumor microenvironment, as both macrophages and granulocytes are potential sources that could be detected in tumors from IL-2/ $\alpha$ -CD40-treated mice. NOS2 immunoreactivity was detected in cells that morphologically resembled macrophages only in tumors from IL-2/ $\alpha$ -CD40-treated mice (Fig. 1 B). Using dual-color immunofluorescent microscopy, we found that a population of F4/80<sup>+</sup> macrophages colocalized with intense immunoreactivity for NOS2 expression within the tumor microenvironment of



**Figure 1. IL-2/ $\alpha$ -CD40 induces NOS expression via tumor-associated macrophages.** (A) WT and GKO mice were treated between days 11 and 21 as indicated in the Materials and methods. On day 22, mice were euthanized, and the primary tumor was dissected and homogenized in RIPA lysis buffer. The expression of NOS2 was determined by Western blot analysis. Hypoxanthine phosphoribosyltransferase (HPRT) was used as a loading control. (B and C) Tumors from treated WT mice were dissected on day 22, fixed in formalin, and analyzed by two-color immunofluorescent microscopy (200 $\times$  magnification). NOS2 immunoreactivity was visualized using Alexa Fluor 488 (green). (B and C) F4/80 (B) and GR1 (C) immunoreactivity was visualized using Alexa Fluor 594 (red). In some regions, colocalization of the two colors results in yellow staining. The results are representative of three separate experiments. Bars, 100  $\mu$ m.

IL-2/ $\alpha$ -CD40-treated mice (Fig. 1 B). It is noteworthy, however, that not all NOS2<sup>+</sup> cells costained with F4/80. Although we believe it most likely that this population of cells represents a phagocytic cell that has down-regulated F4/80 expression, as has been reported for activated macrophages exposed to cytokines from lymphoid-rich regions (Ezekowitz and Gordon, 1982), we do not rule out the possibility that the degree of colocalization between F4/80 and NOS2 may also be attributed to the membrane association of F4/80 and cytoplasmic localization of NOS2. Although F4/80<sup>+</sup> macrophages were evident in the tumors from  $\alpha$ -CD40-treated mice, these were NOS2 negative. No F4/80 immunoreactivity was detectable in tumors from vehicle control (VC; not depicted) or IL-2 (Fig. 1 B)-treated mice. Gr1<sup>+</sup> granulocytes were also present in tumors from IL-2/ $\alpha$ -CD40-treated mice (these appear as much smaller orange-stained cells in the two-color overlay); however, these were primarily localized in NOS2-negative areas (Fig. 1 C). Furthermore, the treatment of mice with the macrophage-depleting agent clodronate abrogated NOS2 expression, whereas Gr1-depletion had no effect (Fig. 1, B and C). These data confirm that IL-2/ $\alpha$ -CD40 induces significant NOS2 expression through a process that is dependent on tumor-associated macrophages.

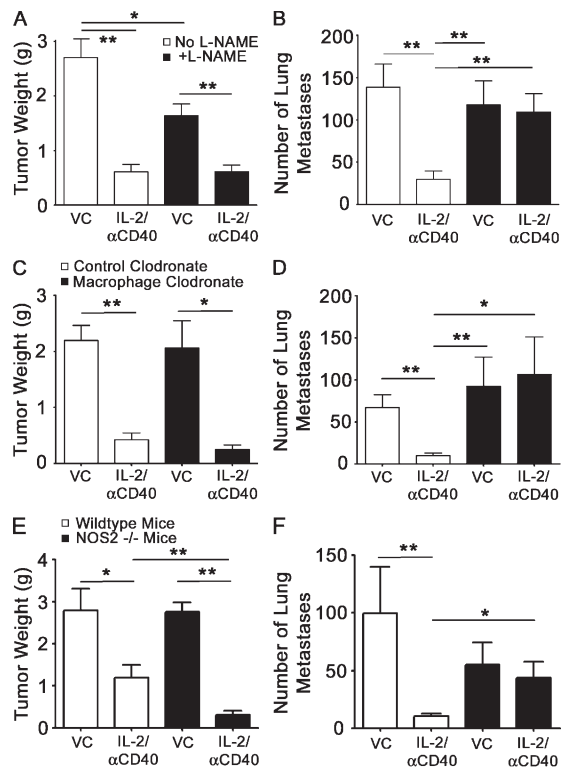
#### Macrophage-dependent NOS expression is required for controlling lung metastases

To confirm the relative importance of macrophage-associated NO expression to tumor progression, we treated tumor-bearing mice with the NOS inhibitor N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) for the duration of therapy. Treatment with L-NAME had no effect on the ability of IL-2/ $\alpha$ -CD40

therapy to reduce primary tumor burden (Fig. 2 A); however, it completely abrogated the ability of this combination immunotherapy to reduce the number of lung metastases (Fig. 2 B). Interestingly, for saline control-treated mice, L-NAME treatment modestly, yet significantly, reduced primary tumor burden by itself (Fig. 2 A). Additionally, macrophage depletion similarly had no effect on the ability of IL-2/ $\alpha$ -CD40 to reduce the size of kidney tumors (Fig. 2 C) but macrophage depletion completely reversed the reduction in the number of lung metastases (Fig. 2 D). To further address the role for NOS2 in tumor progression, we next evaluated primary tumors and lung metastases in WT and NOS2<sup>-/-</sup> mice. Although primary tumors in control-treated WT and NOS2<sup>-/-</sup> mice were indistinguishable, the treatment of NOS2<sup>-/-</sup> mice with IL-2/ $\alpha$ -CD40 resulted in a significantly greater reduction in primary tumor burden, as compared with WT mice (Fig. 2 E). However, the IL-2/ $\alpha$ -CD40-mediated reduction in lung metastases was significantly reversed in NOS2<sup>-/-</sup> mice (Fig. 2 F). Collectively, these data suggest that the immunotherapy-induced NO expression via tumor-associated macrophages is critical for the control of lung metastases but not the size of primary tumors in the kidney.

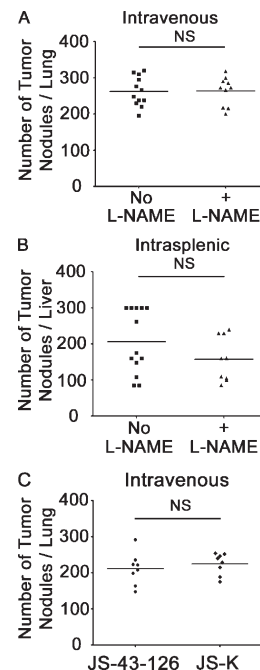
#### NO does not alter the ability of tumors to seed the lung and liver

To determine whether the inhibition of NO was somehow altering the ability of Renca tumor cells to seed different organs, we inoculated mice with tumor cells via intravenous or intrasplenic routes of injection to seed the lungs and liver, respectively. For each route of tumor injection, an equivalent number of tumor nodules were observed in control (no L-NAME) or L-NAME-treated mice (Fig. 3, A and B). This suggests that



**Figure 2. Macrophage-dependent NOS expression is required for controlling lung metastases.** WT and NOS2<sup>-/-</sup> mice were treated as indicated. (A and B) Black bars indicate mice that were treated with L-NAME for the duration of the experiment. (C and D) For clodronate studies, mice received either saline control liposomes (white bars) or macrophage-depleting clodronate (black bars) as detailed in the Materials and methods. (E and F) Black bars indicate results using NOS2<sup>-/-</sup> mice. (A, C, and E) On day 22, mice were euthanized and the primary tumor was dissected and measured. Lungs from these treated mice were collected on the same day and fixed in Bouin's solution. (B, D, and F) The number of lung metastases was counted under a dissecting microscope. In A–D, the pooled results from two experiments are shown and in E and F, the results from one experiment are shown. The error bars indicate the standard error of the means for each treatment group. Each experiment consisted of 10 mice/treatment group. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  for the indicated comparisons.

NOS2 inhibition does not simply act by impairing the ability of Renca to seed and grow in the lung or liver. To further evaluate the role NO might play in regulating distant organ seeding, we treated mice bearing intravenously injected Renca tumor cells with the NO donor drug JS-K. We selected this NO donor because it has proven effective in several xenograft models (Kiziltepe et al., 2007; Maciag et al., 2009), because it releases NO specifically upon activation by glutathione, and this reaction is accelerated by glutathione S-transferase (GST; Shami et al., 2003), and because RCCs express GST (Simic et al., 2009), which should allow for drug targeting to the tumor microenvironment (Shami et al., 2003). Treatment of mice bearing intravenously delivered Renca with either JS-K or the non-NO-releasing analogue JS-43-126 had indistinguishable effects on the number of tumor nodules in the lungs (Fig. 3 C). Collectively, these results indicate that NO blockade or

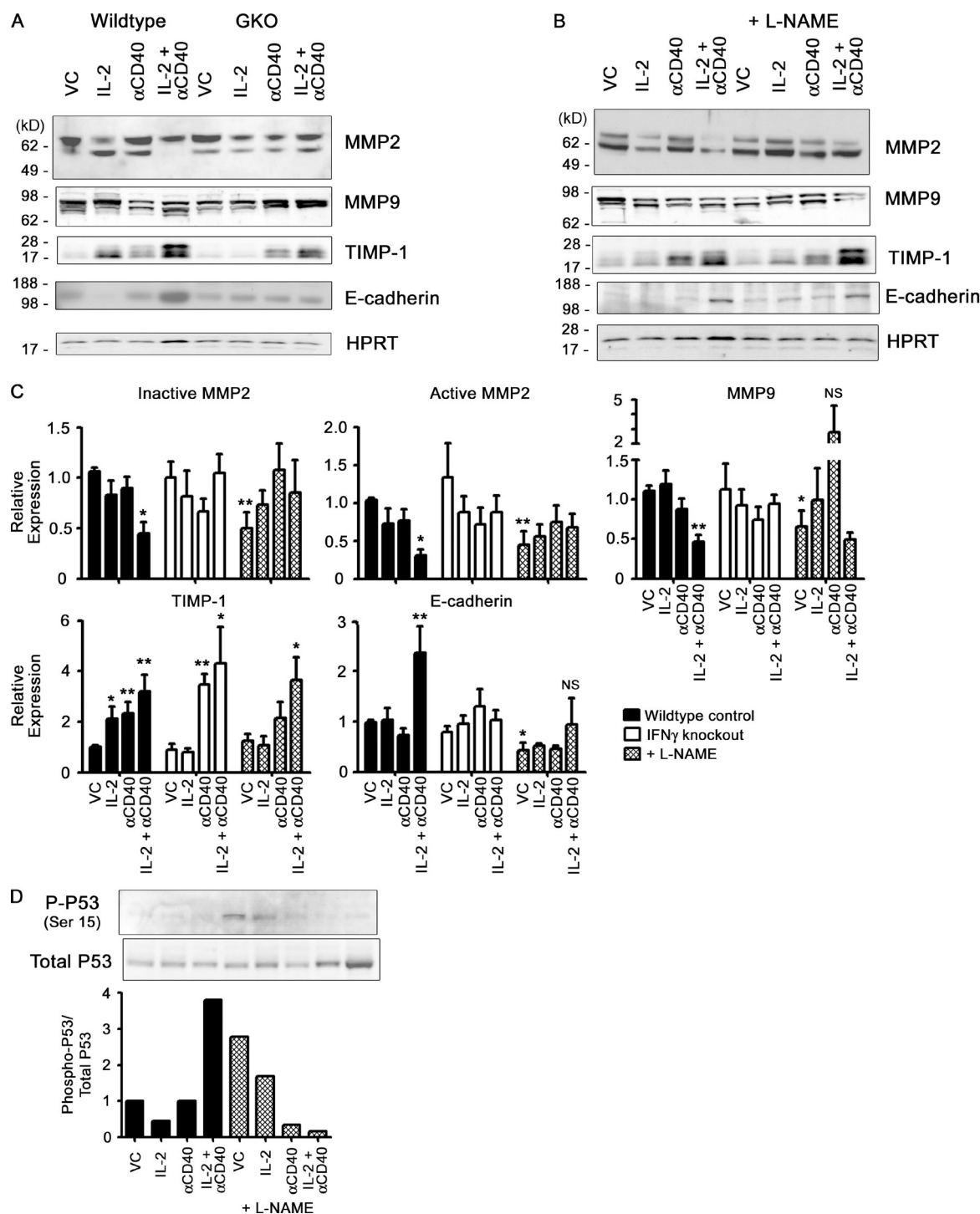


**Figure 3. NO inhibition or stimulation does not impair the ability of tumors to seed the lung and liver.** (A and B) WT mice were inoculated intravenously (A) or intrasplenically (B) with Renca tumor. One-half of the mice were treated with L-NAME for the duration of the experiment. (A–C) WT mice were inoculated intravenously with Renca tumor and treated with either JS-43-126 or JS-K on days 1, 3, 6, 8, 10, and 13 after tumor injection. On day 14, mice were euthanized and the lungs (A and C) and livers (B) were collected and fixed in Bouin's solution. The number of tumor nodules in each organ was determined by counting under a dissecting microscope. Each data point represents the results from an individual mouse and each treatment group contained at least eight mice. The horizontal bars indicate the means for each treatment group. The data are derived from one experiment. The results in A and B are representative of two separate experiments. NS = not significant.

stimulation has no significant impact upon the ability for tumor cells to seed distant organs. Rather, they suggest that NO is an important regulator of the preceding ability of tumor cells to break away from the orthotopically growing primary kidney tumor, thereby allowing them to gain entrance into the vasculature and ultimately metastasize to distant organs.

#### IL-2/ $\alpha$ -CD40 reduces MMP2 and MMP9 expression and increases TIMP-1 and E-cadherin expression within the tumor microenvironment

Our results suggested that the NO-mediated inhibition of metastases was likely occurring proximal to the primary tumor site. Knowing that the expression and activity of MMPs are important mediators of the metastatic process (Liotta and Stetler-Stevenson, 1990), we analyzed MMP2, MMP9, and TIMP-1 expression within the tumor microenvironment after therapy. As shown in Fig. 4, IL-2/ $\alpha$ -CD40 treatment, but not IL-2 or  $\alpha$ -CD40 alone, reduced the expression of both MMP2 and MMP9 within tumors.

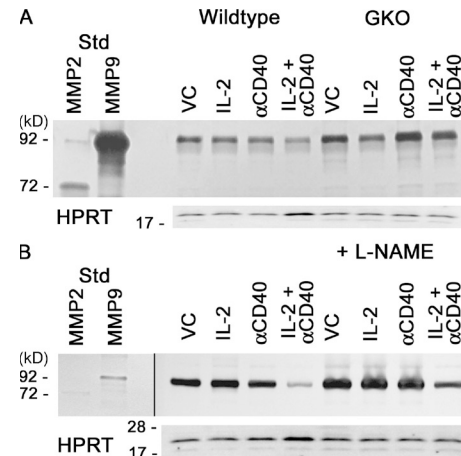


**Figure 4.** IL-2/α-CD40 reduces MMP2 and MMP9 expression and increases TIMP-1 and E-cadherin expression within the tumor microenvironment. (A) WT and GKO mice were treated as indicated. (B) WT mice were treated as indicated with or without continuous L-NAME treatment. On day 22, mice were euthanized and the primary tumor was dissected and homogenized in RIPA lysis buffer. The expression of MMP2, MMP9, TIMP-1, and E-cadherin was determined by Western blot analysis. HPRT was used as a loading control. The results are representative of at least three separate experiments. (C) For each protein, the corresponding band intensity was determined by densitometric analysis. The results were then divided by the band intensity for the corresponding HPRT loading control. For MMP9, the intensity of both major bands was analyzed together. The graphs represent the mean and standard errors for the quantitated results obtained from all experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . The black bars indicate WT mice, the white bars indicate GKO mice, and the hatched bars indicate L-NAME-treated WT mice. (D) The expression of phosphorylated P53 (Ser 15) and total P53 levels was analyzed in treated samples by Western blot analysis. The graph illustrates the band intensity ratio of phosphorylated P53 relative to the corresponding total P53 levels from one experiment. The black and hatched bars indicate WT mice treated in the absence or presence of L-NAME, respectively.

Importantly, the expression of both the inactive form (72 kD) and active form (63 kD) of MMP2 was reduced as compared with control-treated mice. The reduction in MMP2 and MMP9 expression was not observed in GKO mice (Fig. 4 A) or in mice treated with L-NAME (Fig. 4 B), although basal levels of both molecules were significantly lower in L-NAME-treated mice (Fig. 4 C). These findings implicate IFN- $\gamma$ -mediated NO expression in the control of MMP expression within tumors. Furthermore, IL-2/ $\alpha$ -CD40 treatment induced a significant increase in TIMP-1 expression within tumors that was independent of both IFN- $\gamma$  (Fig. 4 A) and NOS2 expression (Fig. 4 B). This latter finding is consistent with the ability of NO to regulate TIMP-1 expression at low concentrations but not higher concentrations (Ridnour et al., 2007). IL-2/ $\alpha$ -CD40 also induced an IFN- $\gamma$ -dependent increase in E-cadherin expression (Fig. 4, A and C). Although IL-2/ $\alpha$ -CD40 mediated a detectable increase in E-cadherin expression in L-NAME-treated mice (Fig. 4 B), this did not reach statistical significance as compared with L-NAME-treated control mice, the latter of which exhibited significantly lower levels of E-cadherin expression, as compared with non-L-NAME-treated mice (Fig. 4 C). Finally, IL-2/ $\alpha$ -CD40 treatment induced P53 phosphorylation, a marker of NO-mediated signaling suggestive of elevated steady-state NO levels which was not observed in the presence of L-NAME (Fig. 4 D). Collectively, these data demonstrate that the high level of NO achieved within the tumor microenvironment by IL-2/ $\alpha$ -CD40 treatment correlates with a potentially reduced metastatic potential of tumor cells through the reduction in local MMP expression and increased expression of molecules involved in the regulation of MMP activity (TIMP-1) and cell adhesion (E-cadherin).

#### IL-2/ $\alpha$ -CD40 reduces MMP9 activity within the tumor microenvironment

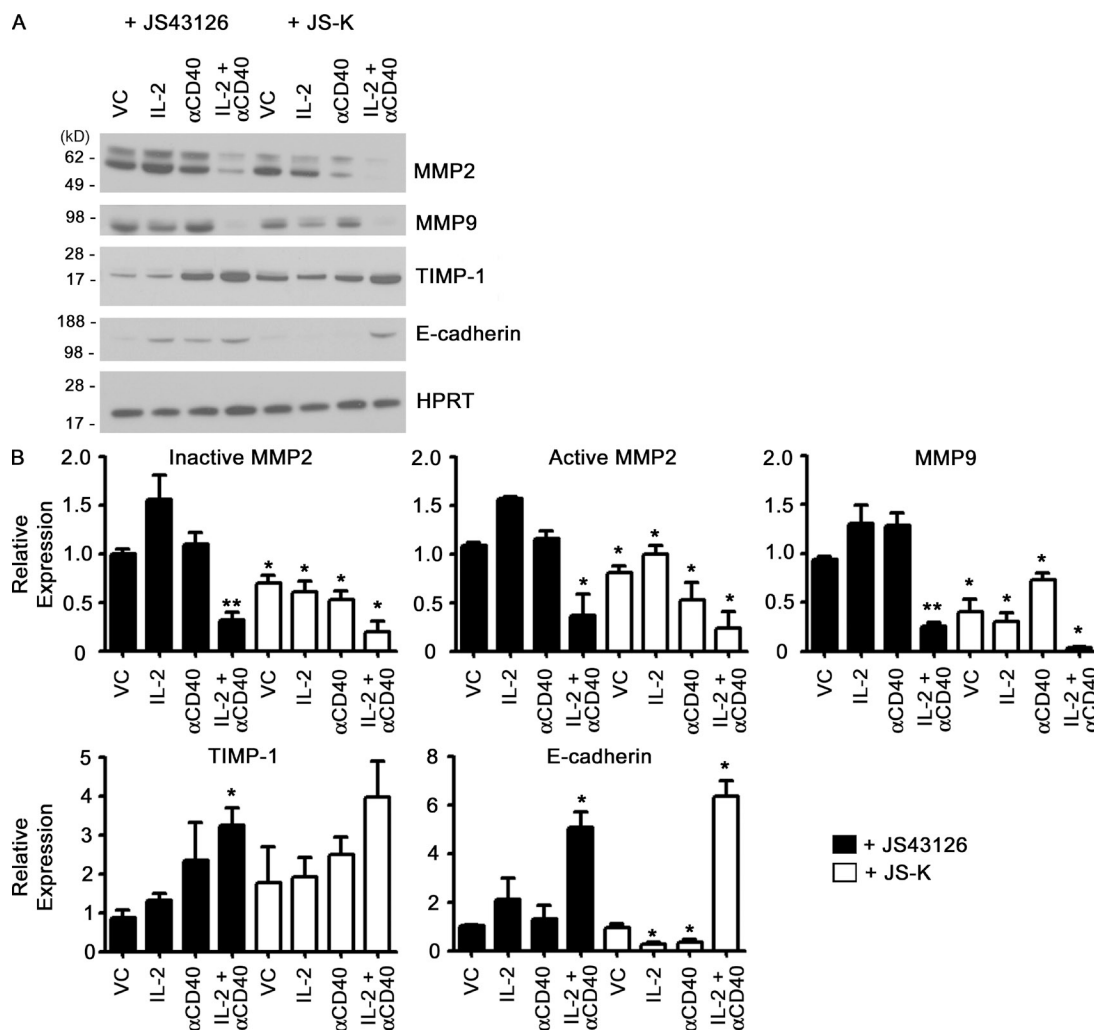
TIMP-1 is a well established endogenous inhibitor of MMP activity, and active MMP2 has also been shown to cleave pro-MMP9 into the active form (Liotta and Stetler-Stevenson, 1990). Furthermore, NO regulates both TIMP-1 and MMP9 (Ridnour et al., 2007). We therefore determined whether MMP enzymatic activity was reduced in response to IL-2/ $\alpha$ -CD40 treatment. By gel zymography, IL-2/ $\alpha$ -CD40 treatment of tumor-bearing mice significantly reduced MMP9 activity within the tumor microenvironment (Fig. 5). Consistent with the previous data, the IL-2/ $\alpha$ -CD40-mediated inhibition of MMP9 activity was not observed in GKO mice and was markedly reduced in mice treated with L-NAME. The partial ability of L-NAME to inhibit MMP9 activity was not surprising because MMP9 can also be inhibited by IFN- $\gamma$  (Duluc et al., 2009). These data collectively demonstrate the ability of IL-2/ $\alpha$ -CD40 combination treatment to induce the IFN- $\gamma$ - and NO-dependent reduction in MMP9 expression and activity within the tumor microenvironment that may, in turn, impair metastatic spread to the lungs.



**Figure 5. IL-2/ $\alpha$ -CD40 induces the IFN- $\gamma$ - and NO-dependent reduction in MMP9 activity within the tumor microenvironment.** (A) WT and GKO mice were treated as indicated. (B) WT mice were treated as indicated with or without continuous L-NAME treatment. On day 22, mice were euthanized and the primary tumor was dissected and homogenized in RIPA lysis buffer. MMP9 activity was determined by gel zymography. Note that because the amount of lysate loaded in each lane was based on the same total protein concentration used for Western blot analyses in the previous figure, the same corresponding HPRT Western blot that was used as a loading control in the previous figure is shown for referencing purposes as a gel loading control. The results are representative of three separate experiments.

#### Treatment with an NO donor, both as a single agent or in combination with IL-2/ $\alpha$ -CD40, reduces MMP-2 and MMP-9, but not TIMP-1 or E-cadherin, expression within tumors

To further confirm the role for NO in regulating MMP-2/-9, TIMP-1, and E-cadherin expression in primary tumors, we evaluated the expression of these molecules after treatment of mice with JS-K or JS-43-126 alone or in combination with IL-2 and/or  $\alpha$ -CD40. By Western blot analyses, we found that treatment with JS-K, alone or in combination with IL-2 or  $\alpha$ -CD40, significantly reduced the levels of both inactive and active forms of MMP-2, as well as MMP-9, as compared with the corresponding mice treated with JS-43-126 (Fig. 6, A and B). Interestingly, when JS-K was used in combination with IL-2 +  $\alpha$ -CD40, MMP-2 levels, and especially MMP-9 levels, were significantly reduced even further, as compared with all other treatment groups. In contrast, treatment with JS-K had no effect on TIMP-1 levels within primary tumors. The basal or IL-2/ $\alpha$ -CD40-induced expression of E-cadherin was also unaffected by JS-K treatment, although it is interesting to note that E-cadherin levels were significantly reduced in tumors from IL-2 + JS-K- and  $\alpha$ -CD40 + JS-K-treated mice (Fig. 6 B). Overall, these findings indicate that the NO donor JS-K is capable of significantly down-modulating MMP-2 and MMP-9, but not TIMP-1 or E-cadherin, expression within tumors. Our data further suggest that NO levels achieved within the tumors can be more effectively targeted using combination therapies for the collaborative reduction of tumor-associated MMP-2 and MMP-9 expression.

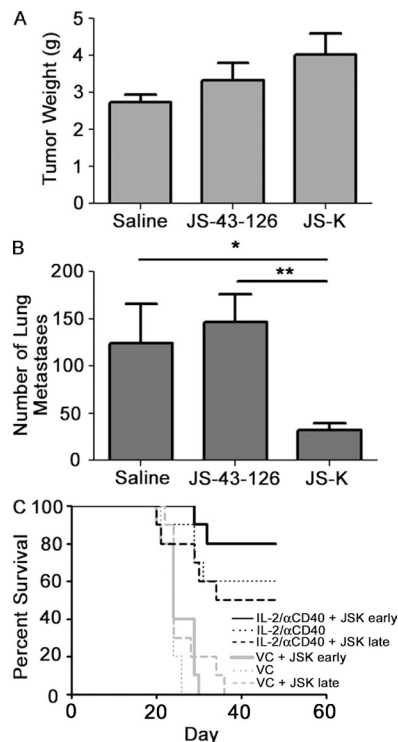


**Figure 6. Treatment with the NO donor JS-K reduces MMP-2 and MMP-9 expression in primary tumors.** WT mice were treated with saline (VC), IL-2, and/or  $\alpha$ -CD40 in combination with either the NO donor JS-K or the non-NO-releasing analogue JS-43-126. On day 22, mice were euthanized and the primary tumor was dissected and homogenized in RIPA lysis buffer. (A) The expression of MMP2, MMP9, TIMP-1, and E-cadherin was determined by Western blot analysis. HPRT was used as a loading control. The results are representative of three separate experiments. (B) For each protein, the corresponding band intensity was determined by densitometric analysis. The results were then divided by the band intensity for the corresponding HPRT loading control. Graphs represent the mean and standard errors for the quantitated results obtained from all three experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . The black bars indicate JS-43-126 control-treated mice and the white bars indicate JS-K-treated mice.

### Treatment with an NO donor reduces lung metastases and improves survival

Our data indicated that endogenous NO expression was a critical component of the immunotherapy-mediated control of lung metastases but not primary tumor burden. To further confirm this, we treated tumor-bearing mice with JS-K or JS-43-126 and evaluated primary tumors and lung metastases. Consistent with our previous results, we found that JS-K treatment had no effect on primary tumor weights (Fig. 7 A) but it significantly reduced the number of lung metastases (Fig. 7 B). When mice were treated with JS-K in combination with either IL-2 or  $\alpha$ -CD40, the anti-tumor effects were no better than single agent treatment (unpublished data). Finally, we evaluated long-term survival of JS-K-treated mice bearing

metastatic disease. Because curative responses after combination therapy using IL-2/ $\alpha$ -CD40 (as well as IL-2/IL-12) require the surgical removal of primary tumors (Wigginton et al., 1996a; Murphy et al., 2003; Weiss et al., 2009), we adapted our tumor model to contrast the treatment with JS-K either before or after the surgical removal of the tumor-bearing kidney on day 11. Mice received JS-K either on days 4, 6, 8, and 10 (JSK early) or on days 13, 15, 18, 20, and 22 (JSK late). Saline control (VC) or IL-2/ $\alpha$ -CD40 treatment always commenced on day 12 and went for two weekly cycles. As compared with the survival results obtained using IL-2/ $\alpha$ -CD40 in the absence of any JS-K treatment, the inclusion of JS-K treatment before nephrectomy slightly enhanced mouse survival, although differences were not statistically significant (Fig. 7 C).



**Figure 7. Treatment with the NO donor JS-K reduces lung metastases and improves survival by acting directly on the primary tumor.** (A and B) WT mice were treated with either the NO donor JS-K or the non-NO-releasing analogue JS-43-126. (A) On day 22, mice were euthanized and the primary tumor was dissected and measured. (B) Lungs from these treated mice were collected on the same day and fixed in Bouin's solution. The number of lung metastases was counted under a dissecting microscope. The pooled results from two experiments each consisting of 10 mice/treatment group are shown. \*,  $P < 0.05$ ; \*\*,  $P < 0.002$  for the indicated comparisons. The error bars indicate the standard error of the means for each treatment group. (C) For studies of tumor progression, some mice were treated with JS-K only on days 4, 6, 8, and 10 (JSK early). All mice received surgical removal of the tumor-bearing kidney on day 11 followed treatment with IL-2/ $\alpha$ -CD40 or saline (VC) beginning on day 12 for 2 wk. Some mice were treated with JS-K on days 13, 15, 18, 20, and 22 (JSK late). Survival analysis was plotted according to the Kaplan-Meier method. The results are from one experiment in which each treatment group contained at least eight mice.

It is noteworthy, however, that no enhancement in survival was observed when mice were treated with JS-K after nephrectomy of the tumor-bearing kidney. Treatment of mice with JS-K in the absence of IL-2/ $\alpha$ -CD40 also failed to improve survival, regardless of when JS-K treatment was initiated. These findings suggest that although JS-K as a single agent appears ineffective, the IL-2/ $\alpha$ -CD40 therapy previously shown to significantly promote survival might modestly be enhanced by JS-K treatment. The requirement for JS-K administration before nephrectomy would be consistent with the conclusion that the NO donor needs to act directly within the primary tumor microenvironment to exert its anti-metastatic effects.

## DISCUSSION

Previously, we showed that the IL-2/ $\alpha$ -CD40 immunotherapeutic regimen results in synergistic anti-tumor responses (Murphy et al., 2003; Weiss et al., 2009). IL-2/ $\alpha$ -CD40, but not treatment with either IL-2 or  $\alpha$ -CD40 alone, significantly reduced primary tumor size and the number of lung metastases and resulted in improved long-term survival. We now demonstrate that the induction of macrophage-dependent NO expression within the tumor microenvironment is a critical component of this successful therapy. This finding builds upon our previous studies showing that tumors from IL-2/ $\alpha$ -CD40-treated mice contain significant infiltration by IFN- $\gamma$ -producing CD8<sup>+</sup> T cells (Weiss et al., 2009), which are also required for the control of metastases because their depletion, even after nephrectomy of primary tumors, impaired survival (Murphy et al., 2003). It is therefore logical to propose a scenario whereby these activated effector cells within the tumor microenvironment contribute to the IFN- $\gamma$ -dependent up-regulation of NOS2 expression via macrophages that we now demonstrate are critical for the control of metastatic spread. Interestingly, the ability of IL-2/ $\alpha$ -CD40 to control primary tumors was independent of NO, whereas the reduction in lung metastases was absolutely dependent on NO. Indeed, the identical reversal of IL-2/ $\alpha$ -CD40-mediated anti-metastatic effects achieved by NOS2 inhibition and macrophage depletion, as well as in NOS2<sup>-/-</sup> mice, strongly supports the conclusion that IL-2/ $\alpha$ -CD40 induces the recruitment of macrophages into the tumor microenvironment where NO production is critical for the control of metastatic spread. Presumably, the reduction in primary tumor burden is dependent on other components achieved by this combination therapy, such as the tumor infiltration by effector CD8<sup>+</sup> T cells that facilitate adaptive immune responses and generate protection from tumor rechallenge (Murphy et al., 2003; Weiss et al., 2009). With respect to the primary kidney tumor, it is important to note that a significant reduction was observed in L-NAME-treated mice but not NOS2<sup>-/-</sup> or clodronate-treated WT mice. Moreover, IL-2/ $\alpha$ -CD40 reduced primary tumors to a much greater degree in NOS2<sup>-/-</sup> mice. Collectively, these data suggest that although NOS2 is central to the control of lung metastases, other NOS isoforms, perhaps derived from tumor-associated vasculature, appear to be more central to the control of primary tumor growth. Because RCC is characterized by a high frequency of metastasis and poor prognosis (Holland, 1973), an improved understanding of the mechanisms whereby this separable process may be potentially controlled is of considerable importance.

Several elegant studies have implicated macrophages in the promotion of metastatic potential (Lin et al., 2001; Qian et al., 2009). In these studies, the expression of macrophage-derived CSF-1 was directly implicated in regulating breast cancer metastasis rather than the growth of primary tumors (Lin et al., 2001). Thus, elevated CSF-1 levels frequently observed in solid tumor patients are explicitly linked with the degree of macrophage infiltration into primary tumors and poor prognosis. Moreover, macrophage depletion blocked

seeding and metastatic growth of tumor cells in the lungs (Qian et al., 2009). However, we believe that some variables may complicate ascribing the role for macrophages in the progression of some tumors. For example, although mice bearing CSF-1-rich tumors in the breast have a dramatic degree of macrophage infiltration, our previous (Weiss et al., 2009) and current (Fig. 1 B) findings indicate that these cells do not represent a major component of the mononuclear cell infiltrate of orthotopic Renca tumors under basal conditions. It remains unclear whether this is a result of tumor- or possibly organ-specific differences between these experimental models. This distinction may, at least in part, explain why macrophage depletion, in the absence of immunotherapy, did not significantly alter the number of lung metastases in our studies.

Although macrophages may frequently be associated with poor outcome, their remarkable heterogeneity and plasticity underscores their promise as cellular targets for improved therapeutic outcome. Our data are consistent with the conclusion that IL-2/ $\alpha$ -CD40, and likely other IL-12-based immunotherapeutic regimens, alters macrophage populations to help induce the establishment of a desirable Th1/M1-like tumor microenvironment. IL-2/ $\alpha$ -CD40 induces significant levels of IL-12 and IFN- $\gamma$  expression, both of which are markers of M1 polarized macrophages. Moreover, within the tumor itself, IL-2/ $\alpha$ -CD40 induces significant infiltration by NK cells, CD8<sup>+</sup> T cells, and macrophages (Weiss et al., 2009). Thus, rather than tumor promotion, in the IL-2/ $\alpha$ -CD40 immunotherapeutic setting, these tumor-associated macrophages display an anti-tumor M1 phenotype, with reduced arginase expression (Weiss et al., 2009) and increased NOS expression. This finding is consistent with a recently proposed model whereby macrophages retain a large degree of plasticity, allowing their effector functions to be reprogrammed by components of their microenvironment. Importantly, IL-12 has emerged as a critical regulator of this process (Watkins et al., 2007). Moreover, our data demonstrate IL-2/ $\alpha$ -CD40-mediated reduced MMP9 expression, which is also consistent with the M1 polarization of macrophages because elevated MMP9 levels have been more closely associated with alternatively activated M2 macrophages (Lolmede et al., 2009). Thus, the observed modulation of MMP9 and TIMP-1 expression within tumors may be a reflection of Th1-polarized macrophages as well as the reduced frequency of MDSC in tumors from IL-2/ $\alpha$ -CD40-treated mice (Weiss et al., 2009). Although the reduced enzymatic activity of MMP9 within tumors might simply be a result of lower MMP9 expression, we also find it noteworthy that the levels of MMP9 activity did not always follow the same trend as that observed for MMP9 expression levels. For example, although L-NAME by itself significantly reduced MMP9 expression, the activity of MMP9 under these same conditions was enhanced, a finding which is consistent with our previous study showing enhanced MMP9 activity after NO inhibition and the biphasic nature of MMP regulation by NO (Ridnour et al., 2007). It is also likely that the elevated TIMP-1 levels we observed are functioning to inhibit MMP9

activity at the protein level because the MMP-independent functions of TIMP-1 are anti-apoptotic, which would otherwise protect the tumor (Liotta and Stetler-Stevenson, 1990). It is noteworthy that several studies have correlated increased MMP expression in tumor samples with metastatic potential in murine studies (Miyake et al., 1999), as well as tumor progression and poor prognosis in RCC patients (Harada et al., 2006; Kawata et al., 2007). Thus, an important immunotherapeutic goal should be the redirection of immune responses such that MMP levels are reduced.

Conflicting results have been reported for NO in cancer progression studies. In certain studies, NOS expression has been positively correlated with tumor invasiveness (Orucevic et al., 1999). Moreover, NO inhibition augmented anti-tumor immunity by interfering with NOS2 activity that is critical for MDSC suppressor function (Serafini et al., 2006b). However, other studies have demonstrated an inverse correlation between cancer progression and NOS expression (for review see Williams and Djamgoz, 2005). Furthermore, NO has been shown to both positively and negatively regulate apoptosis, cell invasion, and angiogenesis (Ambs et al., 1998; for review see Williams and Djamgoz, 2005). The dual nature of NO arises from its concentration-dependent ability to regulate numerous cellular processes that may impact metastatic potential. In tumors, different concentration thresholds of NO appear to elicit a discrete set of signal transduction pathways (Thomas et al., 2004). Low concentrations of NO promoted the growth, invasion, and metastasis of murine mammary tumors (Jadeski et al., 2000). Recently, it has also been shown that low levels of NO result in a transient increase in ERK phosphorylation and a signaling cascade resulting in increased angiogenesis, cell migration, adhesion, and proliferation that may be consistent with the promotion of tumor growth (Thomas et al., 2004). In contrast, high NO levels have been shown to suppress tumor growth and metastasis (Dhar et al., 2003; Le et al., 2005). Consistently, the high levels of NO-induced P53 phosphorylation and events associated with reduced angiogenesis, cell migration, adhesion, and proliferation each correlate with reduced tumor progression (Ambs et al., 1998; Thomas et al., 2004). Our data are consistent with this biphasic model which has been proposed for NO (Ridnour et al., 2006). Under basal control-treated conditions, where low levels of NO are expected, we observed a significant reduction in tumor growth after treating mice with an inhibitor of NO alone. In the context of a strong immunotherapeutic regimen such as IL-2/ $\alpha$ -CD40, we observed significant increases in NOS expression as well as increased phosphorylation of P53 within tumor lysates, which serves as an important indicator that high levels of NO have been achieved. Importantly, our results suggest that the high levels of NO achieved within the tumor microenvironment after IL-2/ $\alpha$ -CD40 treatment are critical for the suppression of tumor metastasis. We demonstrate that this is closely associated with the IFN- $\gamma$ - and NO-dependent modulation of MMP2, MMP9, and TIMP-1 expression and activity (Ridnour et al., 2007).

NO-releasing prodrugs have been used to treat coronary disease; however, their systemic administration may result in dose-limiting vasodilation and hypotension. As a result of the relevant role that NO plays in modulating angiogenesis, tumor cell growth, and sensitization to apoptosis, these agents have also shown considerable promise for tumor immunotherapy (Bonavida et al., 2008). Two NO-releasing vasodilating drugs, isosorbide mononitrate (ISMN) and isosorbide dinitrate (ISDN), inhibited tumor growth and metastasis in the Lewis lung carcinoma model in mice, in association with an inhibition of angiogenesis (Pipili-Synetos et al., 1995). However, the NO donor that we successfully used, JS-K, does have a major advantage over other NO-inducing drugs that warrants further consideration for its use in anti-tumor studies. JS-K was specifically designed to overcome the undesirable vasodilation and hypotension side effects (Shami et al., 2003). JS-K mediates the relatively slow release of NO in microenvironments rich in glutathione, such as many tumor microenvironments including RCC (Simic et al., 2009). JS-K was shown to inhibit breast cancer invasion *in vitro* (Simeone et al., 2008). We now show for the first time that JS-K significantly inhibited lung metastasis *in vivo*. This finding, collectively with our observation that NOS2 inhibition reversed lung metastasis but had no effect on the growth of primary tumors, indicates that the two anti-tumor effects (primary and metastatic) of the IL-2/ $\alpha$ -CD40 immunotherapy are separable and supports strongly the role for macrophage-dependent NO expression in the control of metastatic spread to the lungs. Interestingly, the anti-metastatic benefits of JS-K treatment required an intact tumor because these effects were abolished when the tumor-bearing kidney was resected. Furthermore, the early administration of JS-K, followed by IL-2/ $\alpha$ -CD40 therapy, resulted in slightly enhanced survival, as compared with IL-2/ $\alpha$ -CD40 alone, which would further be consistent with this model. We also found that the inhibition of NOS2 had no effect on the ability of Renca tumors to seed either the lungs or liver when administered via intravenous or intrasplenic routes, respectively. Collectively, these findings strongly suggest that NO prevents metastatic spread by acting directly at the primary tumor site. Indeed, our data suggest that NO donors will likely not provide clinical benefit in patients that have their primary tumors surgically removed. The clinical use of NO donors such as JS-K may thus be more appropriate in cases where surgical removal of primary tumors is not possible or, potentially, in combination with other therapies for the control of tumor recurrence. Conversely, it could be interesting to consider the possibility that some patients regularly taking iNOS inhibitors for other conditions (e.g., erectile dysfunction) might show slower primary tumor growth but an enhanced propensity for metastatic dissemination.

Within the primary tumor, we demonstrate several critical molecular changes in response to IL-2/ $\alpha$ -CD40-induced NO expression that may inhibit the metastatic process. To our knowledge, our study is the first to demonstrate, in an *in vivo* model of metastatic disease, the ability for high levels of NO

expression within the tumor microenvironment to regulate the expression and activity of MMP and E-cadherin and relate this to the metastatic potential of a progressing tumor. Specifically, we noted that IL-2/ $\alpha$ -CD40-induced NO expression increases E-cadherin and reduces MMP expression specifically within the primary tumor microenvironment. E-cadherin is an adhesion molecule that is involved in cell-cell adhesion and responsible for maintenance of epithelial tissue. The loss of E-cadherin expression has been associated with an increased risk for metastasis in human tumors (Oka et al., 1993). Moreover, the treatment of mice with an NO donor also significantly reduced MMP-2 and MMP-9 expression within tumors. After cell detachment from the primary tumor, their invasion of the host stroma is a prerequisite for metastasis (Liotta and Stetler-Stevenson, 1990). In this regard, the levels and activity of MMP2 and MMP9 directly correlate with the invasion and metastatic potential of tumor cells. Many studies have used multivariate analysis to demonstrate that the MMP/E-cadherin ratio is an independent prognostic indicator of overall patient survival for many solid tumors, including RCC (Kuniyasu et al., 1999; Slaton et al., 2001; Fukata et al., 2005; Campos et al., 2006; Pettaway et al., 2008). Thus, IL-2/ $\alpha$ -CD40 immunotherapy should achieve a low MMP/E-cadherin ratio, the most favorable outcome, via both a reduction in MMP expression and activity and a concomitant increase in E cadherin expression in primary tumors. We believe that the relative levels of MMP and E-cadherin expression within the tumor microenvironment represent an important target and molecular readout for the successful immunotherapy of metastatic diseases.

Collectively, our data demonstrate the feasibility of using combination immunotherapy for the control of metastases. The localized expression of NO specifically within the tumor microenvironment serves as an important molecular readout, as this is critical for the regulation of MMP activity and adhesion molecule expression that underlies the inhibition of the metastatic process. The tumor-targeted delivery of JS-K further highlights the potential for context-dependent use of NO agonists in anti-tumor therapies.

## MATERIALS AND METHODS

**Mice.** BALB/c WT mice were obtained from the Animal Production Area of the National Cancer Institute (NCI) at Frederick, MD. BALB/c GKO and NOS2<sup>-/-</sup> mice were obtained from The Jackson Laboratory. PCR primers for genotyping GKO mice were 5'-ATCGACAAGACCGGCTTC-CATCCG-3' and 5'-TCAGCGCAGGGGCGCCCGTTCTTT-3'. PCR primers for genotyping NOS2 mice were 5'-ACATGCAGAATGAGT-ACCGG-3', 5'-TCAACATCTCCTGGTGGAAC-3', and 5'-AATATGC-GAAGTGGACCTCG-3'. Mice (8–10 wk of age) were used in accordance with an approved protocol by the NCI-Frederick Institutional Animal Care and Use Committee.

**Cells and reagents.** The renal adenocarcinoma of BALB/c origin (Renca) was passaged intraperitoneally as previously described (Williams et al., 1981). Recombinant human IL-2 (Teceleukin) was obtained from the NCI. Agonist rat anti-mouse CD40 (clone FGK115B3) was purified from ascites as previously described (Murphy et al., 2003; Weiss et al., 2009). Endotoxin was <1 EU/mg of antibody, as determined by chromogenic Limulus Amebocyte Lysate kit (QCL-1000; Cambrex). Purified rat IgG was purchased from Jackson ImmunoResearch Laboratories. L-NAME was purchased from

Sigma-Aldrich. Clodronate (Cl<sub>2</sub>MDP) and saline control liposomes were purchased from Roche (van Rooijen and van Nieuwmege, 1984). The NO-releasing pro-drug JS-K and non-NO-releasing analogue JS-43-126 were prepared as previously published (Saavedra et al., 2001).

**In vivo tumor models and treatments.** For the orthotopic tumor model, 10<sup>5</sup> Renca cells were injected under the kidney capsule of BALB/c WT or GKO mice. In some experiments, mice were inoculated with 7.5 × 10<sup>4</sup> Renca cells via intravenous (tail-vein) or intrasplenic (followed by immediate splenectomy) routes of injection. Some mice were treated throughout the duration of the experiment with 0.5 g/liter L-NAME in their drinking water (L-NAME/water was replaced every other day). Mice treated with IL-2 received 300,000 IU i.p. twice a day on days 11, 15, 18, and 21 after tumor injection. Mice treated with anti-CD40 received 65 µg i.p. once on days 11–15 and 18–21 after tumor injection. For macrophage depletion studies, mice were injected with 200 µl clodronate or saline control liposomes i.p. on days 11 and 18 after tumor injection. Flow cytometric analysis was used to determine that clodronate treatment resulted in the removal of 80–90% of the tumor-associated monocyte/macrophages. Mice treated with JS-K or JS-43-126 were injected i.p. with 4 µmol/kg, three times/wk beginning on day 11. On day 22, mice were euthanized, primary tumors were collected in saline, lungs were fixed in Bouin's solution, and lung metastases were counted under a dissecting microscope.

In survival studies, mice were treated with JS-K on the indicated days. All mice received a unilateral nephrectomy of the tumor-bearing kidney on day 11, followed by treatment with IL-2 and/or anti-CD40, beginning on day 12 for two weekly cycles. Mice were monitored for tumor progression.

**Immunofluorescent microscopy.** Tumors were fixed in 4% formalin and sectioned in paraffin. Deparaffinized slides were blocked with 2% BSA/PBS and incubated with anti-F4/80 (Invitrogen), anti-GR1 (BD), anti-NOS2 (Abcam), or isotype-matched negative controls, followed by incubation with fluorescently conjugated secondary antibodies (Dako). A digital camera (DXM1200; Nikon) attached to a microscope (Eclipse E400; Nikon) equipped with epifluorescence illumination was used for high-power (200× magnification) fluorescent microscopy. Fluorescent excitation and emission filter combinations were used to image Alexa Fluor 488 (excitation source 480 nm/emission filter 505 nm) and Alexa Fluor 594 (excitation source 540 nm/emission filter 565 nm).

**Western blot analysis.** Primary tumors were dissected cleanly away from as much normal kidney tissue as possible and collected in sterile saline. They were snap-frozen in a dry ice/ethanol bath and stored at −70°C until use. Tumors were homogenized using a GentleMACS Dissociator (Miltenyi Biotec) in 10 ml RIPA buffer containing Halt protease inhibitor (Thermo Fisher Scientific). Lysates were clarified by centrifugation and the total amount of protein was quantitated (Thermo Fisher Scientific). Total protein (10 µg) was electrophoresed on 4–12% gradient polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen). Blots were probed using one of the following antibodies: NOS2 (Cayman Chemicals), MMP2 (Santa Cruz Biotechnology, Inc.), MMP9 (Santa Cruz Biotechnology, Inc.), TIMP-1 (DAKO), phospho-P53 (Cell Signaling Technology), P53 (Santa Cruz Biotechnology, Inc.), E-cadherin (Santa Cruz Biotechnology, Inc.), or HPR1 (Santa Cruz Biotechnology, Inc.), followed by an incubation with HRP-conjugated secondary antibodies and visualization using ECL (Invitrogen).

**Gel zymography.** MMP activity was qualitatively examined by gel zymography (Kleiner and Stetler-Stevenson, 1994). Based on the total protein content as indicated in the Western blotting section, 2 µg of protein in each lysate was prepared in 2× loading buffer without β-mercaptoethanol. Samples were electrophoresed on precast 10% polyacrylamide gels containing 1 mg/ml of gelatin. The gels were washed and incubated in renaturing and developing buffers according to the manufacturer's recommendation, stained with Simply blue, and then destained with a 30% methanol/10% acetic acid solution. All reagents were purchased from Invitrogen.

**Statistical analysis.** Statistical differences were analyzed using a Mann-Whitney *U* test (Prism; GraphPad Software, Inc.). Significance was indicated by *P* < 0.05 values.

We thank Dr. Joseph E. Saavedra for supplying JS-K and JS-43-126. We thank Donna Butcher of the Pathology and Histotechnology Laboratory at National Cancer Institute (NCI) Frederick for her invaluable expertise and assistance with immunofluorescent microscopy. We thank Drs. John Ortaldo and Giorgio Trinchieri for discussions and critically reviewing this manuscript.

This project was funded in whole or part by the Intramural Research Program of National Institutes of Health (NIH)/NCI and with Federal funds from the NCI, NIH under R01-CA-95572 (W.J. Murphy) and SAIC Contract # HHSN261200800001E.

The authors declare no competing financial interests.

Submitted: 6 April 2010

Accepted: 15 September 2010

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