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Decoy Receptor 3 Enhances Tumor Progression via Induction of Tumor-Associated Macrophages

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Tumor-associated macrophages (TAMs) are the major component of tumor-infiltrating leukocytes. TAMs are heterogeneous, with distinct phenotypes influenced by the microenvironment surrounding tumor tissues. Decoy receptor 3 (DcR3), a member of the TNFR superfamily, is overexpressed in tumor cells and is capable of modulating host immunity as either a neutralizing decoy receptor or an effector molecule. Upregulation of DcR3 has been observed to correlate with a poor prognosis in various cancers. However, the mechanisms underlying the DcR3-mediated tumor-promoting effect remain unclear. We previously demonstrated that DcR3 modulates macrophage activation toward an M2-like phenotype in vitro and that DcR3 downregulates MHC class II expression in TAMs via epigenetic control. To investigate whether DcR3 promotes tumor growth, CT26-DcR3 stable transfectants were established. Compared with the vector control clone, DcR3-transfectants grew faster and resulted in TAM infiltration. We further generated CD68 promoter-driven DcR3 transgenic (Tg) mice to investigate tumor growth in vivo. Compared with wild-type mice, macrophages isolated from DcR3-Tg mice displayed higher levels of IL-10, IL-1ra, Ym1, and arginase activity, whereas the expression of IL-12, TNF-α, IL-6, NO, and MHC class II was downregulated. Significantly enhanced tumor growth and spreading were observed in DcR3-Tg mice, and the enhanced tumor growth was abolished by arginase inhibitor N-ω-hydroxy-L-norarginine and histone deacetylase inhibitor sodium valproate. These results indicate that induction of TAMs is an important mechanism for DcR3-mediated tumor progression. Our findings also suggest that targeting DcR3 might help in the development of novel treatment strategies for tumors with high DcR3 expression.

We previously demonstrated that DcR3 modulates the differentiation and activation of macrophages in vitro. DcR3-treated macrophages display attenuated phagocytic activity, with downregulated CD14 and CD16, as well as impaired production of proinflammatory cytokines in response to LPS (33). Recently, we further demonstrated that DcR3 downregulates MHC-II expression in macrophages via epigenetic control, and histone deacetylase (HDAC) inhibitor restores MHC-II expression by reversing DcR3-mediated deacetylation of the promoter of transcription factor CIITA (34). However, whether DcR3 promotes tumor growth and progression via the induction of TAMs in vivo remains to be elucidated. Because the mouse genome does not contain DcR3, CT26 stable clones overexpressing DcR3 and CD68 promoter-driven DcR3-transgenic (Tg) mice were generated to address this question. Evaluation of the influences of DcR3 on TAMs and the subsequent influences on tumor progression was carried out.

Materials and Methods

DcR3 stable transfectants

CT26 murine colon adenocarcinoma cells were transfected with pcDNA3DcR3 or pcDNA3 vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and selected in the presence of G418 (400 μg/ml; Sigma-Aldrich, St. Louis, MO). Each stable clone was confirmed for the expression and secretion of DcR3 by Western blot analysis of protein lysate and ELISA of culture supernatant. Tumor growth was evaluated by inoculating anti-mouse class II (E-9), anti-mouse CD68 promoter (forward primer, 5'-CGG CCA GAT GGA ATT GCC ACG-3'; reverse primer, 5'-TCT AGA CAA GGA GCA TTA GGG CCG TG-3') and subsequently subcloned into XbaI-cleaved pcDNA3-CD68. The 4.7-kb CD68-DcR3 cassette was excised by restriction enzymes HindIII and BstAI and microinjected into fertilized eggs of C57BL/6 (H-2b) females. The potential Tg founder mice were screened by PCR using primers annealing to the CD68 promoter (forward primer, 5'-GAG GTG GCT AGA GCT GAG GCC-3') and DcR3 (reverse primer, 5'-CAG GCA CAG CAG CGA GGC-3'). DcR3 expression was confirmed by Western blot analysis and serum ELISA. All mice were bred and housed at the laboratory animal center, and the studies were approved by the institutional animal care and usage committee of the Animal Care and Use Committee of the National Yang-Ming University.

Tumor models in Tg mice

CT26 cells, derived from BALB/c (H-2b) mice, were used for the establishment of mouse tumor models. DcR3-Tg and wild-type (WT) C57BL/6 (H-2b) mice were crossed with BALB/c (H-2d) mice to generate F1-DcR3-Tg mice and ELISA of culture supernatant. Tumor growth was evaluated by inoculation of each well of a 24-well plate overnight, followed by washing with fresh medium to remove nonadherent cells. To detect the production of arginase, NO, and cytokines, macrophages were incubated with LPS (1 μg/ml) for 24 h before measurement.

Flow cytometry and ELISA

Flow cytometry was used to assess the expression of cell surface markers. Cells were harvested and washed twice with FACS staining/washing buffer (0.1% NaN₃, 1% FCS in PBS), followed by incubation with mAbs. Stained cells were analyzed using a FACS Calibur system, and data were analyzed using FlowJo software (TreeStar, Ashland, OR). ELISA detection kits (R&D Systems, Minneapolis, MN) were used to analyze TNF-α, IL-1ra, IL-6, IL-10, IL-12, and DcR3 levels in mouse sera or macrophage culture supernatants. Serum samples were prepared from 50–100 μl peripheral blood taken from mouse tails. All samples were stored at −20°C before cytokine detection by ELISA, and each sample was tested in triplicate.

Gelatin zymography

Conditioned medium in nonreducing loading buffer was fractionated on a 10% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was washed in 2.5% Triton X-100 to remove SDS and incubated overnight in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 10 mM CaCl₂ at 37°C. Then, the gel was stained with Coomassie blue. Enzyme-digested regions were identified as white bands on a blue background.

Arginase assay and NO assay

Arginase activity was assessed in cell or tissue lysates indirectly by measuring urea concentration generated by the arginase-dependent hydrolysis of l-arginine (35). Briefly, cells were lysed with 50 μl 0.1% Triton X-100, followed by the addition of 50 μl 1 mM Tris-HCl (pH 7.5) and 50 μl 10 mM MnCl₂ and heated at 55°C for 10 min. Arginase hydrolysis was conducted by incubating 50 μl the lysates with 50 μl l-arginine (0.5 M [pH 9.7]) at 37°C for 60 min, followed by the addition of 400 μl stopping solution ([H₂SO₄/H₃PO₄/H₂O = 1/3/7, v/v/v]). 50 μl 9% isonitrosopropiophenone in ethanol (Sigma-Aldrich) and incubation at 95°C for 45 min. Urea concentration was measured at 540 nm.

NO production was determined by measuring nitrite concentration in macrophage culture supernatant using the Griess reaction (36). Briefly, 50 μl macrophage culture supernatant was transferred to each well of a 96-well plate and reacted with an equal volume of Griess reagent (1% salicylamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H₃PO₄) for 5 min at room temperature. The absorbance was determined at 540 nm.

Statistical analysis

Values were expressed as the means ± SD of at least three experiments. Data were analyzed using the Student t test with the Statistical Package of Social Sciences software version 12.0 (SPSS, Chicago, IL); p values < 0.05 were considered statistically significant.

Results

DcR3 induced TAM filtration and promoted tumor growth

To examine the influence of DcR3 on tumor growth in vivo, we performed DcR3-overexpression experiments in mouse CT26 cells that had no background DcR3 expression. Cells were transfected with pcDNA3 vector (CT-26C) or pcDNA3DcR3 plasmid. Two DcR3 stable clones (CT26-D7 and CT26-D24) were generated, and...
intracellular DcR3 was found to be greater in CT26-D7, as determined by Western blot analysis (Fig. 1A). Secretory DcR3 was determined in culture supernatant by ELISA, which revealed high DcR3 secretion in CT26-D7 (DcR3 high), low secretion in CT26-D24 (DcR3 low), and no secretion in CT-26C (Fig. 1C). When inoculated s.c. in BALB/c mice, the tumor growth rate of CT26-D7 was faster than that of CT26-D24 and CT26-C (Fig. 1C). Moreover, DcR3 expression level correlated with infiltration of F4/80+ cells in tumor masses (Fig. 1D). To rule out the roles of T cells and B cells in modulating tumor growth, tumor cells were inoculated s.c. in T cell-deficient nude mice (Fig. 1E) and T and B cell-deficient NOD-SCID mice (Fig. 1F), respectively. We found that CT26-DcR3 transfectants grew faster than did CT26-C, and the growth rate was in accord with the DcR3 levels in stable clones (CT26-D7 > CT26-D24 > CD26-C). These results suggested that secretory DcR3 in the tumor microenvironment can enhance TAM infiltration and promote tumor growth.

Generation and characterization of CD68 promoter-driven DcR3-Tg mice

To explore the systemic effects of DcR3 on TAMs and tumor growth, DcR3-Tg mice driven by CD68 promoter were generated on a C57BL/6 background. Insertion of the CD68-DcR3 transgene into murine genomes was confirmed by PCR, which displayed a 320-bp DNA fragment with an expected size from the DcR3-Tg mice genomic DNA (Fig. 2A). Western blot analysis showed a 33-kDa protein from the splenocyte lysates of DcR3-Tg mice (Fig. 2B), which we also observed in phosphoglycerate kinase promoter-driven DcR3-Tg mice in a previous study (37). Based on DcR3 serum level, DcR3-Tg mice were denoted as DcR3 low (50–300 ng/ml) and DcR3 high (300–900 ng/ml) (Fig. 2C). Flow cytometry analysis of peripheral WBCs showed that DcR3 was detectable in CD68+ mononuclear cells but not in B cells (B220+), CD4+ or CD8+ T cells, or Ly-6G+ neutrophils (Fig. 2D). Immunohistochemical staining revealed positive DcR3 expression in resident macrophages of the lung, liver, and spleen of DcR3-Tg mice (Fig. 2E). Despite a high serum level of DcR3, DcR3-Tg mice showed normal growth and organ development, without detectable disease, during the 1.5-y observation period.

DcR3 modulated macrophage differentiation in DcR3-Tg mice

To examine the influence of DcR3 on macrophage differentiation in vivo, peritoneal macrophages were harvested for flow cytometry analysis on day 5 after i.p. thioglycolate injection. Compared with WT littermates, the expression of MHC-II was downregulated, whereas the expression of MHC class I, CD80, and CD206 remained unchanged in DcR3-Tg mice (Fig. 3A). ELISA demonstrated that IL-10 was 3-fold higher, whereas IL-12 was suppressed to an undetectable level in DcR3-Tg mice after LPS stimulation (Fig. 3B). Moreover, downregulated TNF-α and IL-6 with upregulated IL-1ra was also observed (Fig. 3C). It is interesting to note that arginase activity increased by >2-fold (Fig. 3D), whereas NO production after LPS stimulation decreased by 26% in DcR3-Tg mice (Fig. 3E). Furthermore, Ym1 expression was increased in peritoneal macrophages of DcR3-Tg mice, as revealed by Western blot analysis (Fig. 3F). These results indicated that DcR3 could modulate macrophage differentiation into a M2-like phenotype.

Subcutaneous tumor growth was enhanced by DcR3

We next investigated whether DcR3 could modulate tumor growth in DcR3-Tg mice. CT26 cells (1 × 10⁵) were inoculated s.c. to observe tumor growth in F1-DcR3 and F1-WT littermates. Enhanced tumor growth was first observed (Fig. 4A, 4B) in F1-DcR3 mice compared with F1-WT littermates. Subcutaneous tumors growing up to 2500 mm³ were harvested from F1-DcR3 mice or F1-WT littermates for subsequent analyses. We found that both the expression and activity of arginase were upregulated in F1-DcR3 mice (Fig. 4C, 4D). TAMs were further purified for the analyses of arginase activity and NO and cytokine production.
Increased arginase activity and attenuated reactive NO production were observed in TAMs of F1-DcR3 mice, and cytokine production also showed a pattern similar to that of peritoneal macrophages, with downregulated TNF-α and IL-6 and upregulated IL-1ra (Supplemental Fig. 1). Compared with F1-WT mice, Ym1 expression was significantly increased in tumors isolated from F1-DcR3 mice, as determined by Western blot analysis. However, the expression level of MMP2 and MMP9 was not consistent between tumors isolated from F1-DcR3 and F1-WT mice (Fig. 4E).

A syngenic C57BL/6 model was established to further confirm the influence of DcR3 on tumor growth. B16-F10 melanoma cells (H-2b) were inoculated s.c. in C57BL/6 DcR3-Tg mice (H-2b), and enhanced tumor growth was observed in DcR3-Tg mice compared with WT mice (Supplemental Fig. 2A). These observations suggest that DcR3 can enhance tumor growth via the induction of TAM infiltration and the modulation of TAM differentiation in vivo.

DcR3-enhanced tumor spreading in vivo

We further investigated whether DcR3 could enhance tumor spreading in vivo. Because CT26 tumor cells did not metastasize after s.c. inoculation, tumor cells were injected via tail vein to evaluate their differential colonization ability in lung. CT26 cells (5 × 10⁴) were injected via tail veins to observe lung nodule formation. Compared with F1-WT littermates, gross lung nodule formation increased significantly in F1-DcR3 mice 3 wk after tail vein injection (Fig. 5A). Both the number and volume of lung nodules were increased (Fig. 5B, 5C) and confirmed by microscopy with H&E staining (Fig. 5D). In addition, tail vein injection of B16-F10 cells (5 × 10⁴) was performed in a syngenic C57BL/6 model. Compared with WT mice, significant increases in the size and number of lung nodules were observed in DcR3-Tg mice (Supplemental Fig. 2B, 2C). These results suggest that DcR3 not only promotes s.c. tumor growth, but also enhances tumor spreading to distant sites.

DcR3-enhanced tumor growth was abolished by nor-NOHA and VPA

Because increased arginase activity in TAMs was associated with enhanced tumor growth, we investigated whether inhibition of arginase activity could antagonize the DcR3-mediated tumor-promoting effect. Daily i.p. injections of arginase inhibitor nor-NOHA (40 mg/kg/d) were given after s.c. implantation of CT26 cells. As shown in Fig. 6A and 6B, nor-NOHA treatment suppressed arginase activity and substantially inhibited tumor growth (80% reduction at day 20 postinoculation) in F1-DcR3 mice compared with F1-WT mice. To clarify whether nor-NOHA influences MHC-II expression, peritoneal macrophages harvested from F1-DcR3 and F1-WT mice were incubated in medium supplemented with PBS or nor-NOHA (100 μM) for 48 h. As shown in Fig. 6C, nor-NOHA did not alter the level of MHC-II expression. This suggests that suppression of tumor growth by nor-NOHA is unrelated to MHC-II expression.

Because DcR3 downregulates MHC-II expression in TAMs via epigenetic regulation, and HDAC inhibitor trichostatin can restore the expression in vitro (34), we investigated whether HDAC inhibitor could inhibit DcR3-mediated tumor enhancement in vivo. Because of the toxicity of trichostatin, another U.S. Food and
Drug Administration-approved HDAC inhibitor (VPA) was used for the animal study. After daily i.p. injections (30 mg/kg/d) of VPA for 5 d, MHC-II (I-Ad) expression was partially restored in the peritoneal macrophages of F1-DcR3 mice (Fig. 6D), and the enhanced tumor growth by DcR3 was suppressed to a level similar to that in F1-WT littermates (Fig. 6E). In addition, arginase activity in s.c. inoculated tumor cells in VPA-treated mice was evaluated. As shown in Fig. 6F, arginase activity was not suppressed by VPA in arginase assays. This observation supports the argument that upregulation of arginase activity may not be via epigenetic regulation by histone deacetylation, and suppression of tumor growth by VPA is not attributed to the alteration of arginase activity in F1-DcR3 mice. Therefore, epigenetic regulation to induce TAM differentiation is one crucial mechanism for DcR3-mediated tumor growth enhancement, and it can be one novel therapeutic target for tumors with high DcR3 expression.

Discussion
Smoldering inflammation has recently been considered a hallmark of cancer with an important role in tumor initiation and progression (38–40), and TAMs are the major component of tumor infiltratory infiltration (7, 8). Thus, understanding the key factors that modulate TAM differentiation is crucial for elucidating the mechanisms underlying TAM-mediated tumor-promoting effects. It has been known that macrophages/TAMs are heterogeneous, plastic cells with different functions and cytokine production in response to various microenvironmental signals, including LPS, IFN-γ, IL-4, or IL-10 (2–4). In this study, we demonstrated that DcR3 modulates macrophage/TAM differentiation in vivo, with higher levels of IL-10, IL-1ra, Ym1, and arginase activity and lower levels of IL-12, TNF-α, IL-6, NO, and MHC-II. Moreover, the DcR3-modulated macrophages contribute to tumor progression.

DcR3 is a soluble factor upregulated in the sera of cancer patients and those suffering from acute respiratory distress syndrome, and it is associated with a poor prognosis (29, 31, 41). We previously found that DcR3 modulates macrophage differentiation and activation, as well as suppresses MHC-II expression in TAMs via epigenetic regulation in vitro (33, 34). The current study investigated whether DcR3 promotes tumor growth via the modulation of TAMs in vivo. Compared with the vector control clone (CT26-C), DcR3 stable transfectants (CT26-D7, CT26-D24) grew faster in
immunocompetent (BALB/c) mice, as well as in T or B cell-deficient (nude and NOD-SCID) mice. In addition, greater M2-like F4/80+ cell infiltration was observed in tumors overexpressing DcR3 when inoculated in BALB/c mice. These observations support the argument that DcR3-modulated TAMs contribute to the protumoral effect. To avoid the potential interference of immune responses against human DcR3 after inoculating DcR3 stable clones in mice, CD68 promoter-driven DcR3-Tg mice were generated to confirm the DcR3-mediated effects on macrophages. It was reported that CD68 promoter can induce a high level of transgene expression in macrophages in vitro and in vivo (42). We further confirmed the tissue-specific expression of DcR3 in CD68 promoter-driven DcR3-Tg mice by flow cytometry, as well as the circulating DcR3 in DcR3-Tg mice serum (Fig. 2). Thus, the CD68 promoter-driven DcR3-Tg mice are very helpful to elucidate the systemic immunomodulatory effect of DcR3 in vivo.

In contrast to the actin promoter-driven DcR3-Tg mice, which develop lymphadenopathy and systemic lupus erythematosus-like syndrome at 5–6 mo with a short lifespan (43), the CD68 promoter-driven DcR3-Tg mice had a normal lifespan without the \[\text{FIGURE 4. Enhanced s.c. tumor growth by DcR3. CT26 cells (1 \times 10^5) were inoculated s.c. in F1-DcR3 and F1-WT littermates. Tumor tissues with similar size (2500 mm^3) were harvested for analyses. (A) A dose-dependent enhancement of tumor growth was observed in F1-DcR3 mice (}\ n = 5\). DcR3 low: <300 ng/ml; DcR3 high: >300 ng/ml, by serum DcR3 level. *\ p < 0.05. (B) Tumors (denoted by two arrows) were larger in F1-DcR3 mice. (C) Increased arginase production by immunostaining (original magnification ×400). (D) Arginase assay determined in 150 μg tumor protein lysate revealed a dose-dependent increased arginase activity in F1-DcR3 mice. (E) Increase in Ym1 expression in tumors isolated from F1-DcR3 mice by Western blot analysis.\]

\[\text{FIGURE 5. Enhancement of CT26 tumor spreading in DcR3-Tg mice. (A) Lung metastatic nodules 3 wk after tail vein injections of 5 \times 10^4 CT26 cells in F1-DcR3 or F1-WT mice. Arrows indicate the metastatic nodules. (B) Dose-dependent increase in the number of metastatic lung nodules in F1-DcR3 mice (}\ n = 5\). (C) The average diameter of the lung nodules increased with DcR3 level. Each point represents the average diameter of all lung nodules of a single mouse. (D) H&E staining of mouse lung nodule tissue sections (original magnification ×40 [left panels] and ×100 [right panels]). Scale bars, 1 mm (left panels) and 0.5 mm (right panels). *\ p < 0.05, **\ p < 0.01.\]
Supplemented with PBS or nor-NOHA 100 nM D (30 mg/kg/d) of VPA for 5 d.

We also observed that DcR3 increased the mechanism of DcR3 contributing to tumor progression in vivo via the study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech- enceing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mech-

It is interesting to note that tumor masses isolated from F1-DcR3 mice express higher levels of Ym1 (Fig. 4E), a marker of alternative- ly activated macrophages (45). This suggests that increased infiltration of Ym1 + TAMs is under the influence of DcR3 (Fig. 1D). However, the expression of MMP2 and MMP9 is not up-regulated in either the peritoneal macrophages or tumor masses isolated from DcR3-Tg mice. Thus, the activities of MMP2 and MMP9 might not play an important role in the DcR3-mediated protumoral effect.

Tumor-driven immune suppression has been advocated to be crucial for tumor progression and metastasis, whereas the mechan- isms are heterogeneous and remain to be elucidated (17, 44). We (37, 46, 47) and Han et al. (43) demonstrated that DcR3 acts as a neutralizing “decoy receptor,” as well as an “effector” to skew host immunity toward Th2-dominant responses by influ- encing the activation and differentiation of dendritic cells. In this study, we demonstrated an additional immunomodulatory mechan- ism of DcR3 contributing to tumor progression in vivo via the induction of TAMs. We also observed that DcR3 increased the population of Gr1 + CD11b + myeloid-derived suppressor cells in both peripheral blood and tumor-infiltrating cells (Supplemental Fig. 3). Therefore, these observations suggest that DcR3 possesses pleiotropic immunomodulatory properties and may be an important factor driving the evolution of immune suppression in cancer patients.

Upregulation of arginase activity and downregulation of MHC-II expression are characteristics of DcR3-modulated macrophages. Arginine metabolism through the arginase pathway in macrophages has been demonstrated to enhance tumor growth by providing polyamines that are required for cell replication (48). In contrast, downregulation of MHC-II expression in macrophages can suppress adaptive antitumor immune responses by limiting their ability to present tumor-associated Ags to T cells (6). Because enhanced tumor growth was abolished by arginase inhibitor (nor-NOHA) and HDAC inhibitor (VPA), this observation further indicates that DcR3-modulated TAMs play crucial roles in enhanc- ing tumor progression. Furthermore, nor-NOHA did not influence MHC-II expression, and suppression of tumor growth by VPA was also independent of arginase activity (Fig. 6).

Our results may offer several novel therapeutic implications. Ar- ginase inhibition might not be as effective in humans because up-regulation of arginase activity has not been observed in human TAMs (49). Given that restoration of MHC-II expression by the HDAC inhibitor VPA suppressed tumor growth in F1-DcR3 mice, epige- netic regulation modifying TAM differentiation may be another important tumor-promoting mechanism that can targeted. It would be interesting to investigate whether the HDAC inhibitor, which has been applied in clinical cancer therapy (50), can synergistically enhance the efficacy of standard chemotherapy in patients whose tumors exhibit a high level of DcR3 expression. Although MMP2 and MMP9 are not critical for the protumoral effect in this study, the MMP inhibitor doxycycline (30 mg/kg/d) still contributes to the suppression of tumor growth in DcR3 mice (Supplemental Fig. 4). Thus, other MMPs may also contribute to DcR3-mediated en- hancement of tumor growth. Because DcR3 is almost undetectable in normal individuals, directly targeting it in patients with high DcR3 expression by neutralizing Abs or cancer vaccines may be another novel treatment concept and is worthy of further investigation.
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Disclosures
The authors have no financial conflicts of interest.

References

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Figure S1. Phenotype of TAMs isolated from F1-DcR3 Tg mice. (A) Arginase activity and (B) NO production before (basal level) and after LPS stimulation was determined using 15 μg TAM lysate. (C) Determination of TNF-α, IL-6 and IL-ra levels in culture supernatants of purified TAMs by ELISA. *, p < 0.05; **, p<0.01; ***, p<0.001. (n=5).
Figure S2. Enhanced local growth and distant colonization of B16-F10 (H-2b) melanoma cell line in C57BL/6 DcR3-Tg mice. (A) Tumor growth rate after subcutaneous inoculation (1x10^5 cells) in DcR3-Tg mice and WT littermates. (B) Tumor nodules in lung tissue at day 19 post tail vein injection (5x10^4 cells). (n=5). (C) Gross view of lung metastatic nodules (black arrows). *, p < 0.05.
Figure S3. Increase of MDSCs in peripheral blood and tumor tissues after subcutaneous inoculation of CT26 in F1-DcR3 mice. Determination of MDSCs (Gr-1$^+$CD11b$^+$) in peripheral blood of F1-DcR3 mice (DcR3) and F1-WT littermates (WT) before (A) and after (B) subcutaneous inoculation of CT26 cells (1 x 10$^5$/per mice) by flow cytometry. (C) Determination of MDSCs in tumor mass after subcutaneous inoculation of CT26 cells (1 x 10$^5$/per mice). Samples were harvested from F1-DcR3 and F1-WT mice when tumor size reaches 2500 mm$^3$. 
Figure S4. Inhibition of subcutaneous tumor growth by doxycycline in F1-DcR3 mice.
Subcutaneous inoculation of CT26 cells (1 x 10^5/per mice) in F1-DcR3 mice (DcR3) and F1-WT littermates (WT) without feeding, or fed with doxycycline (30 mg/kg/day) in drinking water. Doxycycline was prepared freshly every 48 hours.