**Intratumoral delivery of mTORC2-deficient dendritic cells inhibits B16 melanoma growth by promoting CD8⁺ effector T cell responses**

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**ABSTRACT**

Dendritic cells (DC) play a pivotal role in the induction and regulation of immune responses. In cancer, DC-based vaccines have proven to be safe and to elicit protective and therapeutic immunological responses. Recently, we showed that specific mTORC2 (mechanistic target of rapamycin complex 2) deficiency in DC enhances their ability to promote Th1 and Th17 responses after LPS stimulation. In the present study, bone marrow-derived mTORC2-deficient (Rictor⁻⁻) DC were evaluated as a therapeutic modality in the murine B16 melanoma model. Consistent with their pro-inflammatory profile (enhanced IL-12p70 production and low PD-L1 expression versus control DC), intratumoral (i.t.) injection of LPS-activated Rictor⁻⁻ DC slowed B16 melanoma growth markedly in WT C57BL/6 recipient mice. This antitumor effect was abrogated when Rictor⁻⁻ DC were injected i.t. into B16-bearing Rag⁻⁻ mice, and also after selective CD8⁺ T cell depletion in wild-type hosts in vivo, indicating that CD8⁺ T cells were the principal regulators of tumor growth after Rictor⁻⁻ DC injection. I.t. administration of Rictor⁻⁻ DC also reduced the frequency of myeloid-derived suppressor cells within tumors, and enhanced numbers of IFNγ⁺ and granzyme-B⁺ cytotoxic CD8⁺ T cells both in the spleens and tumors of treated animals. These data suggest that selective inhibition of mTORC2 activity in activated DC augments their pro-inflammatory and T cell stimulatory profile, in association with their enhanced capacity to promote protective CD8⁺ T cell responses in vivo, leading to slowed B16 melanoma progression. These novel findings may contribute to the design of more effective DC-based vaccines for cancer immunotherapy.

**KEYWORDS**

CD8⁺ T cells; cell activation; dendritic cells; mTORC2; melanoma

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**INTRODUCTION**

DC are professional antigen-presenting cells that shape immune responses by linking innate and adaptive immunity. Their ability to regulate differentiation, activation, and proliferation of specific T-cell subsets, makes them an attractive target in cancer immunotherapy approaches. Indeed, in a large number of clinical trials performed over the last 20 y, DC-based vaccines have proven to be safe (i.e. low toxicity and no impairment in quality of life), and capable of eliciting tumor-specific immunological responses. Recent reviews have focused on the operational discrepancy between the apparent immunogenicity of such vaccines and, thus far, their only limited therapeutic efficacy. These clinical results suggest that DC-based therapies are biologically active in cancer patients, but that their impact on disease can still be refined and improved upon.

The mammalian/mechanistic target of rapamycin (mTOR) pathway plays a key role in regulating mRNA translation, protein synthesis, glucose metabolism, lipid synthesis and autophagy in various cells, including immune cells. mTOR performs these functions in two independent complexes: mTORC1 and mTORC2. mTORC1 phosphorylates the translational factors S6 kinase-1 (S6K1) and 4E-binding protein-1 (4EBP1), regulating different cell processes in response to nutrients and/or growth factors, while mTORC2 phosphorylates Akt, protein-kinase Cz and SGK1 (serum and glucocorticoid-regulated kinase 1), leading to regulation of the actin cytoskeleton. Inhibitors of mTORC1, including rapamycin and its derivatives everolimus and temsirolimus, have been evaluated previously as antitumor agents, with everolimus recently approved for the treatment of breast cancer patients (in combination with exemestane) and patients with advanced pancreatic neuro-endocrine tumors. Novel dual mTORC1/2 inhibitors are currently being studied, both translationally and clinically, as...
anticancer agents, i.e., in hepatocellular carcinoma patients.

Interestingly, during melanoma progression, a number of alterations have been identified in major components of the mTOR pathway, including PI3K (phosphoinositide 3-kinase) mutation, PTEN (phosphatase and tensin homolog) loss of function, and Akt, S6K1, 4EBP1 and elf4E (eukaryotic initiation factor 4E) overexpression. Recently, Damsky and colleagues have reported that activation of mTORC1 and mTORC2 is required for oncogene-induced senescence evasion in human melanomas with BRAF mutations, leading to malignant transformation. Therefore, mTOR is also an appealing therapeutic target for the development of novel treatment options for patients with melanoma. Indeed, dual mTORC1/2 inhibitors have been reported to improve the antitumor efficacy of PI3K or MEK (mitogen-activated protein kinase) inhibitors in melanoma patients. We have shown recently that DC deficiency in mTORC2 results in increased production of pro-inflammatory cytokines (mainly IL-12p70 and IL-23) after TLR4 ligation, leading to enhanced T-helper 1 (Th1) and Th17 responses when compared to control DC. Given that IL-12p70-producing DC vaccines enhanced T-helper 1 (Th1) and Th17 responses when compared to control DC marked by IL-12p70 and IL-23, we hypothesized that Rictor−/− DC might regulate cancer growth when delivered into the tumor microenvironment (TME). To test this possibility, 1 × 10^6 LPS-stimulated control or Rictor−/− DC were injected into established B16 melanomas in WT B6 mice on days 7 (when mean tumor size is approximately 50 mm^2) and 14 post-tumor inoculation, and tumor growth monitored subsequently. As shown in Fig. 2, i.t. injection of LPS-activated Rictor−/− DC markedly slowed B16 melanoma growth when compared to tumor-bearing mice treated with control DC or PBS. This therapeutic effect was not observed when the same number of LPS-Rictor−/− DC were instead injected distal to tumors (i.e. intraperitoneally; data not shown), supporting the need to deliver the treatment within the immediate vicinity of the diseased tissue.

### Results

**Rictor−/− DC exhibit pro-inflammatory properties**

We next investigated whether slowed tumor growth could be ascribed to superior migratory ability of LPS-Rictor−/− DC (versus control DC) to draining lymph nodes or the spleen, where protective antitumor T cell cross-priming would be expected to occur. To test this possibility, B16 melanoma-bearing mice were injected i.t. with LPS-stimulated and CFSE-labeled control or Rictor−/− DC, and the inguinal lymph nodes, spleens and tumors recovered for analysis on day 3 post-treatment. Our results show that injected LPS-Rictor−/− DC were recovered at lower frequencies (Fig. 3A) and lower numbers within the tumors when compared to control DC (Fig. 3B), suggesting that Rictor−/− DC may have migrated more efficiently to tumor-draining lymph nodes. However, we observed similar numbers of migratory control and Rictor−/− DC in the inguinal lymph nodes of treated animals (Fig. 3C), suggesting that Rictor−/− DC did not have a migratory advantage to secondary lymphoid tissues when compared to control DC.

**Intratumoral (i.t.) delivery of LPS-activated rictor−/− DC slows B16 melanoma growth**

Given that Rictor−/− DC secrete markedly elevated levels of IL-12p70 compared with WT controls, and that this type-1 cytokine plays an important role in promoting protective immune responses within the tumor milieu, we hypothesized that Rictor−/− DC might regulate cancer growth when delivered into the tumor microenvironment (TME). To test this possibility, 1 × 10^6 LPS-stimulated control or Rictor−/− DC were injected into established B16 melanomas in WT B6 mice on days 7 (when mean tumor size is approximately 50 mm^2) and 14 post-tumor inoculation, and tumor growth monitored subsequently. As shown in Fig. 2, i.t. injection of LPS-activated Rictor−/− DC markedly slowed B16 melanoma growth when compared to tumor-bearing mice treated with control DC or PBS. This therapeutic effect was not observed when the same number of LPS-Rictor−/− DC were instead injected distal to tumors (i.e. intraperitoneally; data not shown), supporting the need to deliver the treatment within the immediate vicinity of the diseased tissue.

**I.t. delivered rictor−/− DC show similar migratory potential to draining lymphoid tissue, but promote reduced frequencies of MDSC within the TME**

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Superior cross-priming of protective T cells or the action of these effector cells in the TME could also occur if the injected LPS-Rictor−/− DC impaired regulatory cell function in vivo. MDSC have been shown to suppress antitumor T cell immune responses in the TME. We assessed the impact of i.t.-delivered DC on the prevalence of MDSC in the TME. As shown in Fig. 3D, the frequency of MDSC (CD11b°Gr1°) in the Rictor−/− DC-treated mice was reduced significantly (> 70%) when compared to control DC-treated mice, whereas no significant difference was found in overall CD11c+ cell content. This result indicates that the injected Rictor−/− DC may reduce the incidence and regulatory action of MDSC in the TME.
I.t. delivery of Rictor\textsuperscript{−/−} DC exerts antitumor activity in a contralateral (distal) tumor site

To test whether i.t. Rictor\textsuperscript{−/−} DC could have an effect at a distant tumor site, mice with established tumors on their right and left flanks were injected i.t. on the right with PBS or control DC or Rictor\textsuperscript{−/−} DC on days 7 and 14 post-tumor implantation, while tumors on the left of each animal were left untreated. Tumor sizes were monitored longitudinally every 3–4 d until the termination of the experiment. The results show that Rictor\textsuperscript{−/−} DC had an antitumor effect both in the treated tumor (right side) and the untreated tumor (left side), when compared with both PBS and control DC (Fig. 4). This result suggests that Rictor\textsuperscript{−/−} DC have the potential to reduce the growth of a tumor distant from the site of therapeutic injection.

The therapeutic benefit(s) provided by i.t.-delivered Rictor\textsuperscript{−/−} DC is CD8\textsuperscript{+} T cell-dependent

Given that DC can cross-present antigens acquired from dead/dying cancer cells to T lymphocytes, promoting the development of tumor-specific immune responses,\textsuperscript{28} we next assessed whether the therapeutic effect of Rictor\textsuperscript{−/−} DC on melanoma growth was mediated by activated T cells. To test this, control or Rictor\textsuperscript{−/−} DC were injected i.t. into B16 melanomas established in Rag\textsuperscript{−/−} mice (deficient in B and T cells), and melanoma growth monitored over 21 d. Our results show no difference in melanoma growth between mice treated with either control DC or Rictor\textsuperscript{−/−} DC (Fig. 5A), indicating the importance of B and/or T cells in controlling melanoma growth in Rictor\textsuperscript{−/−} DC-treated recipients. As humoral immune responses have generally been found to be poor at regulating B16 tumor growth,\textsuperscript{29} we focused further attention on discriminating the roles of T cell subsets in our protective therapy model. WT mice bearing established day-7 B16 melanomas were injected i.t. with Rictor\textsuperscript{−/−} DC in combination with specific monoclonal antibodies to deplete CD8\textsuperscript{+} or CD4\textsuperscript{+} T cells, or NK cells in vivo. These studies revealed that depletion of CD8\textsuperscript{+} T cells, but not CD4\textsuperscript{+} T cells or NK cells, mitigated the therapeutic benefit associated with i.t.-delivered Rictor\textsuperscript{−/−} DC (Fig. 5B). These findings suggest that
therapy-induced CD8\(^+\) T cells are the primary host immune cell population responsible for regulating tumor growth after treatment with Rictor\(^{-/-}\) DC.

**Rictor\(^{-/-}\) DC administration promotes increased frequencies of cytotoxic CD8\(^+\) tumor-infiltrating lymphocytes (TIL) in vivo**

We next analyzed whether i.t.-injected Rictor\(^{-/-}\) DC were superior to control DC in priming antitumor T cell responses in vivo. Mice bearing 7-day established B16 melanomas were injected i.t. with \(1 \times 10^6\) Rictor\(^{-/-}\) or control DC, with an identical treatment given 1 week later. Tumors were harvested at day 21 and infiltrating T cells isolated and stained. Although tumors from Rictor\(^{-/-}\) DC-treated mice were smaller than those from untreated and control DC-treated mice, the total numbers of CD4\(^+\) and CD8\(^+\) T cells were similar between control DC- and Rictor\(^{-/-}\) DC-treated animals, and higher than in untreated mice (Fig. 6A). These results suggest that both control and Rictor\(^{-/-}\) DC induce some degree of TIL recruitment, but that only the T cells primed by Rictor\(^{-/-}\) DC are able to regulate tumor growth. We next investigated the phenotype of these therapy-induced TIL. Our results show that CD4\(^+\) TIL from Rictor\(^{-/-}\) DC- or control DC-treated mice displayed similar frequencies of IFN\(\gamma\), IL-17\(^+\) and regulatory (CD25\(^+\)Foxp3\(^+\)) T cell populations (Fig. 6B). However, CD8\(^+\) TIL from Rictor\(^{-/-}\) DC-injected animals displayed higher frequencies of IFN\(\gamma\) and granzyme-B\(^+\) cytotoxic subpopulations versus CD8\(^+\) TIL recovered from controls (Fig. 6C). Based on recent evidence that PD-1 plays a key role in modulating immune-mediated regulation of melanoma growth,\(^{30}\) PD-1 expression was evaluated on CD8\(^+\) TIL. We observed no significant difference between Rictor\(^{-/-}\) DC- and control DC-injected animals in PD-1 expression (Fig. 6C). When taken together, these results suggest that i.t.-delivered Rictor\(^{-/-}\) DC promote increased recruitment and therapeutic action of cytotoxic CD8\(^+\) TIL in vivo.

**I.t.-delivery of Rictor\(^{-/-}\) DC promotes improved activation of antitumor CD8\(^+\) T cells in the periphery**

To evaluate T cell activation in the periphery, spleens were harvested from treated mice at day 21, and isolated CD8\(^+\) T cells subsequently evaluated for tumor reactivity. As shown in Fig. 7A and B, splenic CD8\(^+\) T cells isolated from Rictor\(^{-/-}\) DC-treated mice produced higher levels of IFN\(\gamma\) and granzyme-B in response to stimulation with B16-melanoma-bearing B6 mice. After 3 d, tumors, spleens and tumor-draining inguinal lymph nodes were harvested and cells isolated. (A) Plots show the percentages of CFSE\(^+\) DC recovered from the tumor. (B, C) Absolute numbers of CFSE\(^+\) DC recovered from the tumor (B) and inguinal lymph nodes (C). Box plots show median, 25%- and 75%-quartiles, and both extreme values. (D) Percent CD11c\(^+\) and CD11b\(^+\)Gr1\(^+\) cells in the tumor shown as means + SD for three animals per group. *p < 0.05.
Rictor$^{+/−}$ DC-primed CD8$^{+}$ T cells versus control DC- or no DC-primed CD8$^{+}$ T cells (Fig. 7C). Next, we tested the direct cytotoxicity of these in vivo-primed splenic CD8$^{+}$ T cells against B16 melanoma cells in vitro. T cells were cultured for 18 h with CFSE-labeled B16 melanoma cells and violet-labeled irrelevant control EL4 thymoma cells at different T cell:target cell ratios. The viability of residual B16 and EL4 cells was then analyzed by flow cytometry (Fig. 7D). The results show an increased ability of Rictor$^{+/−}$ DC-primed CD8$^{+}$ T cells to kill B16 tumor cells compared to control CD8$^{+}$ T cells (Fig. 7E). The percentage killing of irrelevant EL4 target cells was < 4% for all conditions, supporting the B16-specific nature of CD8$^{+}$ T cell-mediated cytotoxicity. These results support the conclusion that i.t. delivery of Rictor$^{+/−}$ DC improves the cross-priming of cytotoxic, type-1 anti-melanoma CD8$^{+}$ T cells in the periphery compared to untreated or control DC-treated mice.

Discussion

The main novel findings in this study are as follows: (i) the superior systemic therapeutic efficacy of mTORC2-deficient (vs. control) DC when injected i.t. into mice with 1 or more established B16 melanomas, (ii) the associated enhanced (cross)priming of type-1 cytotoxic (IFN$\gamma$/GzB$^+$) CD8$^{+}$ T cells in secondary lymphoid organs, (iii) treatment-associated enhancement of type-1 cytotoxic CD8$^{+}$ T cell recruitment into the TME and (iv) treatment-associated reduction in MDSC regulatory populations in the TME. Despite the predicted ability of Rictor$^{+/−}$ DC to also prime and polarize tumor-specific CD4$^{+}$ T cell responses in treated mice, treatment benefits were CD4$^{+}$ independent, and the phenotype and functional status of CD4$^{+}$ T cells in the periphery and the TME of Rictor$^{+/−}$ DC-treated mice was indistinguishable from control treated animals. Our current results are consistent with those reported by Amiel et al. where the injection of rapamycin-treated DC pulsed with LPS and OVA (as a vaccine) in OVA-B16 melanoma-bearing mice delayed tumor growth and increased the frequency of Ag-specific CD8$^{+}$ TIL.31 Our findings are also in line with the need to promote enhanced frequencies of type-1 tumor-specific CD8$^{+}$ TIL clinically in order to manifest therapeutic benefit in cancer patients.32–34 Indeed, increased IFN$\gamma$ secretion from tumor-specific CD8$^{+}$ T cells has been strongly correlated with prolonged survival in patients with metastatic melanoma post-immunotherapy.35,36

The ability of DC to cross-prime type-1 cytotoxic CD8$^{+}$ T cell responses has been strongly linked to DC production of IL-12p70.37,38 Furthermore, the clinical efficacy of DC-based vaccines in patients with glioma or melanoma has been strongly associated with the level of IL-12p70 produced by patient-derived DC at the time of their injection.23,39 It has also been

Figure 4. i.t.-delivery of Rictor$^{+/−}$ DC results in a therapeutic antitumor response in a contralateral tumor site. Mice bearing 7-d B16 tumors in each flank, with similar mean total tumor sizes, were injected with 50 µL of PBS or 50 µL of PBS containing 10$^6$ control DC or 10$^6$ Rictor$^{+/−}$ DC on the right flanks. Tumors on the left flank of each animal were left untreated. Animals received an identical treatment on day 14 post-tumor implantation. Treated and untreated tumor sizes (in mm$^2$) for each animal were then monitored longitudinally every 3–4 d, and are reported as means + SD for five animals per group. $p < 0.05$.

Figure 5. Reduction of B16 melanoma growth is dependent mainly on CD8$^{+}$ T cells. (A) Rag$^{1−/−}$ mice bearing day 7 s.c. B16 melanomas were given i.t. injections of 10$^6$ control DC or Rictor$^{+/−}$ DC. DC injection was repeated at day 14 post-tumor inoculation. Tumor growth was monitored every 3–4 d and is shown as means + SD for five animals per group. (B) C57BL/6 mice bearing s.c. B16 melanomas were treated on days 7 and 14 post-tumor inoculation by i.t. injection of 10$^6$ Rictor$^{+/−}$ DC. On days 6, 13, and 20 after tumor inoculation, different groups of mice were injected i.p. with control IgG, anti-CD4, anti-CD8$^+$, or anti-NK Ab to specifically deplete these cell populations in vivo. Tumor growth was monitored every 3–4 d and is reported as mean + SD for five animals per group.
reported previously that i.t. DC expression of IL-12 is necessary for the priming of CD8+ T cell responses in patients with breast cancer. These reports are consistent with our findings that the superior efficacy of Rictor−/− DC appears to be tied to their differential high levels of IL-12p70 production, associated with the preferential cross-priming of type-1 cytotoxic CD8+ T cells in vivo.

The effective migration of DC to the lymph nodes and subsequent activation of antigen-specific T cells is thought to be important for the success of immunotherapy with DC-based vaccines. Curiously, injected Rictor−/− DC did not display a migratory advantage to tumor-draining secondary lymphoid organs when compared with control DC, which was consistent with their very similar levels of CCR7 expression. Yet the few migratory injected Rictor−/− DC appeared better at activating therapeutic type-1 CD8+ T cells than control DC, pointing to the improved function of Rictor−/− DC once present in secondary lymphoid organs. Our finding of inefficient trafficking of injected DC to lymphoid tissues in vivo is highly consistent with clinical reports. In melanoma patients, only approximately 4% of DC injected intradermally reach the draining lymph nodes, with these few DC still sufficient to induce antigen-specific immunologic responses. The fact that most of the injected

**Figure 6.** Rictor−/− DC administration promotes increased frequencies of tumor-infiltrating cytotoxic T cells. C57BL/6 mice bearing s.c. B16 melanomas were injected i.t. at days 7 and 14 post-tumor inoculation with 10⁶ control DC or Rictor−/− DC. At day 21 post-tumor inoculation, tumors were harvested and tumor-infiltrating lymphocytes obtained and stained for the indicated surface markers and intracellular cytokines. (A) Plots show absolute numbers of CD4+ and CD8+ tumor-infiltrating T cells divided by the respective tumor weight. (B) Frequencies of IFNγ+, IL-17+ and CD25+Foxp3+ cells in the total CD4+ T cell population. (C) Frequencies of IFNγ+, granzyme-B+ and PD-1+ cells in the total CD8+ T cell population. Data are shown as means ± SD for three to four animals per group, and two independent experiments. *p < 0.05 and **p < 0.01.
DC remained at the injection site, in our case the tumor, which leads us to hypothesize that the T cell priming may also take place in the TME. Indeed, this has been reported previously for Tbet-engineered DC injected directly into established tumor lesions in mice. Recent evidence suggests that extranodal T cell priming in ectopic or tertiary lymphoid organs correlates with better overall survival of cancer-bearing patients. Therefore, we must be open to the likelihood that it is not necessary for injected DC to migrate to secondary lymph nodes in order to effectively prime tumor-specific T cell responses that yield therapeutic benefit.

Figure 7. Rictor DC administration enhances activation of antitumor CD8+ T cells in the periphery. C57BL/6 mice bearing s.c. B16 melanomas were treated at days 7 and 14 post-tumor inoculation with i.t. injection of 106 control DC or Rictor DC. At day 21 post-tumor inoculation, spleens were harvested and (A, B, C) splenocytes stained with CFSE and then stimulated with irradiated (100 Gy) B16 cells (ratio 10:1 respectively) in the presence of 30 IU/mL recombinant human IL-2 for 5 d in 24-well culture plates. (A, B)Responder T cells were analyzed for CD8+ and intracellular IFNγ and granzyme-B, showing (A) representative plots and (B) means SD for six animals per group reported from three independent experiments performed. (C) Cell-free supernatants of these cultures were analyzed for IFNγ, IL-6 and TNF-α. Box plots show median, 25%- and 75%-quartiles, and both extreme values. (D, E) Splenic CD8+ T cells isolated from untreated (PBS), control-DC- or Rictor DC-treated mice were cultured with CFSE-labeled B16 melanoma cells and violet-labeled irrelevant control EL4 thymoma cells at ratios of 5:1:1, 2:1:1 or 1:1:1 (where a unit of 1 = 5 x 104 cells) for 18 h. Cells were analyzed by flow cytometry to determine the percentage of viable B16 (green) or EL4 (violet) cells, as shown in (D), versus a control consisting of a 1:1 mixture of each of the labeled tumor cell lines in the absence of T cells. (E) Results are reported as means +/- SD of data obtained from five mice/ cohort. Percent killing was determined based on the formula: 100% × [1 – (percentage of viable tumor cells in the presence of T cells/percentage of viable tumor cells in the absence of T cells)]. *p < 0.05, **p < 0.01, ***p < 0.001.
Overall, our findings may contribute to the design of more effective DC-based vaccines and cancer immunotherapies. In this regard, transfection with siRNA specific for Rictor has been performed successfully both in mouse BMDC and in human monocyte-derived DC. Therefore, this strategy could be used to inhibit mTORC2 in patient-derived DC-based cancer immunotherapy approaches. Another strategy to consider would involve the use of dual mTORC1/2 inhibitors, which are currently being studied in translational and clinic studies as potential antitumor agents (ClinicalTrials.gov Identifier: NCT02064608, NCT02403895, NCT02193633). These agents could be used to inhibit mTORC1/2 in DC ex vivo, with subsequent injection of the conditioned DC. Furthermore, vaccines based on mTORC2-deficient DC could be administered in combination with other anticancer therapies that would be anticipated to improve their effectiveness. For example, they could be combined with agents that help to recruit effector cells into the tumor, as has been shown for tyrosine kinase inhibitors, such as dasatinib. Agents that normalize the tumor blood vasculature could also be used in combination with DC-based vaccines. In this regard, drugs targeting vascular endothelial growth factor (VEGF) or its receptor have shown encouraging clinical outcomes in early-phase studies when combined with chemotherapy, demonstrating survival extension in patients with metastatic melanoma. Immune targeting of the NOTCH antagonist delta-like 1 homolog (DLK1) has also shown promising results in normalizing the vasculature in the TME of renal cell carcinoma mice models. Furthermore, while in the present study Rictor−/− DC promoted the loss of MDSC in the TME, the negative impact of Treg on type-1 CD8+ T cell function could likely still serve as a therapeutic impediment. Hence, combined use of Rictor−/− DC with regimens designed to minimize/eliminate Treg numbers/function (i.e., anti-CTLA-4) or to enhance T effector function/survival (i.e. anti-PD1, anti-PDL1, anti-Tim3, adoptive T cell therapy) would be expected to yield even greater treatment-associated benefits in the cancer setting.

In summary, our data show that i.t. injection of LPS-activated, mTORC2-deficient DC markedly slows established B16 melanoma growth in mice in a CD8+ T-cell-dependent manner. Administration of Rictor−/− DC reduces the frequency of MDSC within the tumors, and enhances numbers of INFγ+ and granzyme-B+ CD8+ T cells both in host spleens and tumors. These data indicate that selective inhibition of mTORC2 in DC can enhance the therapeutic efficacy of DC-based vaccines for cancer immunotherapy.

Materials and methods

Mice

Male C57BL/6j (B6) and B6.129S7- Rag1tm1Mom/J (Rag1−/−) mice were purchased from The Jackson Laboratory. CD11c-specific Rictor−/− mice were made by crossing floxed Rictor mice (generously provided by Drs Keunwook Lee and Mark Boothby, Vanderbilt University School of Medicine) with B6 mice expressing CD11c-Cre (The Jackson Laboratory). The genetic background of crossed mice was verified by PCR genotyping and littermates used as negative controls. All studies were performed according to an Institutional Animal Care and Use Committee-approved protocol in accordance with NIH guidelines.

Generation of bone marrow-derived DC

Bone marrow (BM) cells were harvested and cultured to generate DC as described, using mouse rGM-CSF and rIL-4 (both 1000 U/mL; R&D Systems, CAA26822 and P07750). On day 7 of culture, DC were purified using anti-CD11c immunomagnetic beads (Miltenyi Biotec, 130-052-001). Where indicated, the TLR4 ligand LPS (100 ng/mL; *Salmonella minnesota* R595; Alexis Biochemicals, ALX-581-008) was used to stimulate DC for 16–18 h.

B16 therapy model

B6 mice received an s.c. injection of 10⁵ B16 melanoma cells in the right flank on day 0. On day 7, mice were randomized into treatment cohorts (five mice each). 10⁶ control DC or Rictor−/− DC were injected intratumorally (i.t.) in 50 μL of PBS on days 7 and 14 post-tumor inoculation. Tumor size was assessed every 3–4 d and recorded in mm² by determining the product of the largest perpendicular diameters measured by vernier calipers.

For bilateral treatment models, mice were challenged s.c. in each flank with 10⁵ B16 melanoma cells. After 7 d, the animals were randomized into cohorts (five mice/group) with similar mean total tumor sizes, with tumors on the right flanks injected with 50 μL of PBS or 50 μL of PBS containing 10⁶ control DC or 10⁶ Rictor−/− DC. Animals received identical treatment on day 14 post-tumor implantation. Tumors on the left flank of each animal were not treated. Treated and untreated tumor sizes (in mm²) for each animal were then monitored longitudinally every 3–4 d, until the termination of the experiment on day 22 post-tumor inoculation.

Tracking of DC in vivo

Control DC or Rictor−/− DC were stained with CFSE following the manufacturer’s instructions (Vibrant CFDA-SE Cell Tracer Kit; Invitrogen, V12883) and 5 × 10⁶ injected i.t. on day 7 post-tumor inoculation. After 3 d, tumors, spleens and inguinal lymph nodes were harvested and cells isolated and stained for analysis.

In vivo immune cell subset depletion

On days 6, 13, and 20 after tumor inoculation, mice were injected i.p. with purified antibodies (Ab): 50–100 μg rat isotype control IgG (Sigma, I4131), 50 μg anti-CD4⁺ monoclonal Ab (GK1.5; ATCC®, TIB-207), 100 μg anti-CD8⁺ monoclonal Ab (ATCC®, TIB-105), or 50 μg anti-asialo GM1 monoclonal Ab (DAKO, 9861000), as previously described. Ab-mediated depletion was 95% effective for the targeted immune cell subset based on flow cytometric analysis of peripheral blood mononuclear cells obtained by tail venipuncture from treated mice 24 h after Ab administration (data not shown).
Tumor-infiltrating leukocyte isolation from B16 tumors

B16 tumors were carefully excised from the mice and weighed. The tumors were then minced and washed using PBS with 5% fetal bovine serum and 2% EDTA. Thereafter, they were disrupted mechanically in digestion media (pre-warmed RPMI-1640 with 0.25 mg/mL of Liberase TL (Roche, 05401020001) and 0.02 mg/mL of DNAse I (Sigma, D4527)), and incubated in the same media with agitation for 1 h. The cell suspensions were centrifuged at 1500 rpm for 5 min, several times, until the supernatants were clear. The cells were then filtered through a 40 μm mesh and leukocytes recovered using Lympholyte M (Cedarlane Laboratories Limited, CL5035) gradient centrifugation for 20 min at room temperature. After washing, leukocytes were enumerated using trypan blue (Gibco, 15250–06) in a Neubauer counting chamber.

Evaluation of T cell responses against B16 tumors ex vivo

For ex vivo T cell stimulation, spleens were harvested from mice 21 d after tumor inoculation and pooled splenocytes stimulated with irradiated (100 Gy) B16 cells (ratio 10:1 respectively) in the presence of 30 IU/mL recombinant human IL-2 (30 U/mL; R&D Systems, 202-IL-050) for 5 d in 24-well culture plates. The responder T cells were analyzed for surface markers and intracellular cytokines. Cell-free supernatants were stored −80°C until cytokine quantitation.

In vitro flow-based cytotoxicity assay

Splenocytes were harvested on the day of euthanasia from bilateral tumor-bearing mice treated by i.t. injection of PBS, control DC or Rictor−/− DC. CD8+ T cells were then isolated from individual, disaggregated spleens by specific MACS (Miltenyi). After trypsinization to obtain single-cell suspensions, B16 melanoma cells and irrelevant control EL4 thymoma (ATCC) cells were labeled for 30 min at 37°C in culture media containing 100 nM carboxyfluorescein succinimidyl ester (CFSE; Thermofisher) or 1 μM Violet Proliferation Dye 450 (BD Biosciences), respectively. After washing with PBS, T cells and tumor target cells were added to wells of V-bottom (96-well) culture plates (Costar) at T cell:B16 melanoma:EL4 thymoma cell ratios of 5:1:1, 2:1:1 or 1:1:1 (where a unit of 1 = 5 × 10⁴ cells) in a total volume of 200 μL of complete media and further incubated at 37°C for 18 h. Cells were then re-suspended in PBS containing 2% paraformaldehyde and analyzed by flow cytometry to determine the percentage of viable B16 (green) or EL4 (violet) cells versus a control consisting of a 1:1 mixture of each of the labeled tumor cell lines in the absence of T cells. Percent killing was determined based on the formula: 100% × [1 − (percentage of viable tumor cells in the presence of T cells/percentage of viable tumor cells in the absence of T cells)]. Results are reported as means +/− SD of data obtained from five mice/cohort.

Flow cytometric analyses

For assessment of intracellular cytokine expression, T cells were examined after 4–5 h restimulation with PMA (0.5 μg/mL, Sigma-Aldrich, P8139) and ionomycin (1 μg/mL, Sigma-Aldrich, I0634) in the presence of GolgiStop (BD Biosciences, 555029). After extensive washes, cells were stained with appropriate Ab, and fixed/permeabilized (eBioscience, 00-5123-43 and 00-5223-56) prior to intracellular staining. Fluorochrome-conjugated mAbs were purchased from eBioscience, BD Bioscience, Biolegend or Miltenyi Biotec. Appropriately-conjugated, isotype-matched IgGs served as controls. Data were acquired with a LSR II or Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo (TreeStar).

Cytokine quantitation

Cytokines in DC or MLR culture supernatants were quantified by ELISA (eBiosciences) and/or cytometric bead array (CBA; BD Bioscience, 558266) where indicated, following the manufacturer’s instructions.

Statistical analyses

Results are expressed as mean ± 1SD. Significant differences between groups were determined using the Student’s t test or one-way ANOVA test (GraphPad Prism), with p < 0.05 considered significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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