

ORIGINAL RESEARCH

Inhibition of SIRP α in dendritic cells potentiates potent antitumor immunity

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ABSTRACT

Despite their central function in tumor immunity, dendritic cells (DCs) can respond to inhibitory signals and become tolerogenic, curtailing T cell responses *in vivo*. Here, we provide the evidence for an inhibitory function of signal regulatory protein (SIRP) α in DC survival and activation. In tumors from human liver cancer patients, infiltrative DCs expressed elevated levels of SIRP α , which is correlated with the induction of immune tolerance within the tumors. Silencing of SIRP α resulted in a significant increase in the longevity of antigen-pulsed DCs in the draining lymph nodes. In addition, SIRP α controls the activation and output of DCs. Silencing of DC-expressed SIRP α induced spontaneous and enhanced production of IL12 and costimulatory molecules, resulting in more potent cytotoxic T lymphocyte responses, including the eradication of previously established solid tumors. SIRP α exerted such effects, at least in part, via the association and sequestration of p85 subunit of PI3K. Thus, SIRP α is a critical regulator of DC lifespan and activity, and its inhibition might improve the clinical efficacy of DC-based tumor vaccines.

Abbreviations: BMDCs, bone marrow-derived dendritic cells; CCR, chemokine receptor; CFSE, carboxyfluorescein succinimidyl ester; CTL, cytotoxic T lymphocyte; DCs, dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IgSF, immunoglobulin superfamily; MHC, major histocompatibility complex; NF- κ B, nuclear factor- κ B; PBDCs, peripheral blood-derived dendritic cells; PI3K, phosphoinositide 3-kinase; SHP2, SH2-domain-containing protein tyrosine phosphatase 2; SIRP α , signal regulatory protein α ; TLRs, Toll-like receptors; TRANCE, tumor necrosis factor-related activation-induced cytokine; TRP-2, tyrosinase-related protein-2

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Introduction

DCs are characterized by a high capacity for antigen capture and processing, migration to lymphoid organs and the expression of various costimulatory molecules for antigen-specific lymphocyte activation, which endows them with key roles in the adaptive immune system.¹ The unique machinery of DCs makes them ideal natural adjuvants for cancer vaccines. Nevertheless, tumors do not induce an inflammatory response conducive for optimal activation of DCs, and as a result, the ensuing immune response is weak and ineffective.^{2,3}

The immunostimulatory properties of DC-based vaccines are limited by their short lifespan and transient activation state within lymphoid tissues.⁴ The lifespan of activated antigen-bearing DCs has been estimated to be as short as 3 d. Prolonging the lifespan of DCs by means of anti-apoptosis can increase the availability of antigen for T cell stimulation and promote immune responses.⁵⁻⁷ The survival of activated DCs is partly regulated by pathogen-derived molecules, acting through one or more evolutionarily conserved Toll-like receptors (TLRs),⁸⁻¹⁰

as well as T cell-expressed costimulatory molecules, such as CD40 ligand (CD40L)¹¹ and TRANCE (tumor necrosis factor-related activation-induced cytokine) whose anti-apoptotic activities depend on Bcl-2 family members and nuclear factor (NF)- κ B subunits.¹² Although the importance of TLRs and CD40 in DC longevity and activity is well documented, rapid turnover of mature DCs in secondary lymphoid organs suggests that DCs possess several negative feedback mechanisms that might allow them to control the magnitude and duration of adaptive immunity beyond the point of maturation.¹³ Therefore, inhibition of key intrinsic negative regulatory signaling is likely to enable development of a general strategy to augment DC longevity and activity, thus enhancing DC-based vaccines.

SIRP α , a plasma-membrane protein expressed mainly by myeloid cells, including DCs, contains putative tyrosine phosphorylation sites in its cytoplasmic region that conform loosely to inhibitory immunoreceptor tyrosine-based inhibition motifs (ITIMs). These motifs mediate the association of SIRP α with the phosphatase SH2-domain-containing protein

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tyrosine phosphatase 2 (SHP2).¹⁴ The extracellular region consists of three immunoglobulin superfamily (IgSF) domains which bind to either widely expressed transmembrane ligand CD47¹⁵ or soluble ligands, such as the surfactant proteins A and D, which are copious in the lungs.¹⁶ Interaction of CD47 with SIRP α negatively regulates phagocytosis of host cells by macrophages.¹⁷⁻²¹ Consistently, anti-CD47 antibody or engineered SIRP α variants with increased affinity for CD47 can induce potent tumoricidal activity of macrophage by antagonizing CD47 on cancer cells.^{22,23} In lungs, ligation of SIRP α to macrophages by surfactant proteins is required to keep the activity of alveolar macrophages in check, thus preventing damage to the airways caused by pro-inflammatory responses.^{16,24} These data strongly suggest that SIRP α induces inhibitory signals in immune response. Here, we demonstrate that the expression level of SIRP α represents a threshold and self-limiting factor for DC activation, most likely by regulating the lifespan and adjuvant capacity of these cells.

Results

SIRP α is upregulated in tumor-associated DCs

To investigate the role of SIRP α in DC activation, we treated mouse bone marrow-derived DCs (BMDCs) and human peripheral blood-derived DCs (PBDCs) with LPS, CpG and polyI:C and then measured the levels of SIRP α protein. A transient downregulation and a subsequent restoration of SIRP α expression were observed in both cells after stimulation (Fig. 1A and B), suggesting that downregulation of SIRP α is integral in DC activation. We then explored whether SIRP α expression is altered in tumor-associated DCs. The expression levels of SIRP α in infiltrative CD11c⁺ DCs in 10 hepatocellular carcinomas (HCCs) were compared with those in CD11c⁺ PBDCs obtained from the same patients. PBDCs from five normal healthy people were used as a control. In PBDCs from patients with HCC, SIRP α was marginally upregulated compared with SIRP α expression in the PBDC control (Fig. 1C). However, its expression was significantly higher in tumor-associated CD11c⁺ DCs

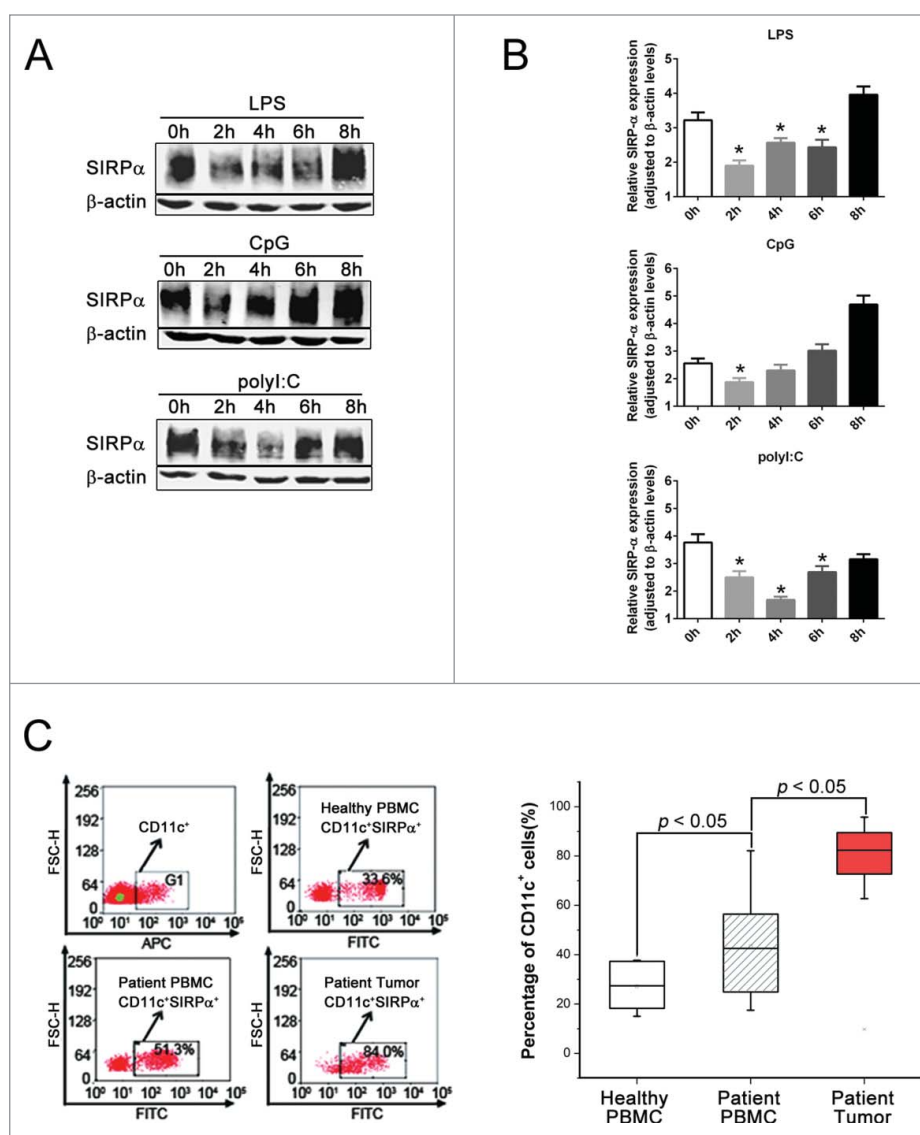


Figure 1. SIRP α is upregulated in tumor-associated DCs. (A & B) Mouse BMDCs from C57BL/6 mice were incubated with 100 ng/mL LPS, 5 μ g/mL CpG or 0.25 μ g/mL polyI:C for the indicated time, and SIRP α protein expression levels were ascertained by Western blotting. (C) FACS analysis of surface expression of SIRP α in CD11c⁺ DCs infiltrated in HCC and in autologous CD11c⁺ PBDCs. PBDCs from five normal healthy people were used as control.

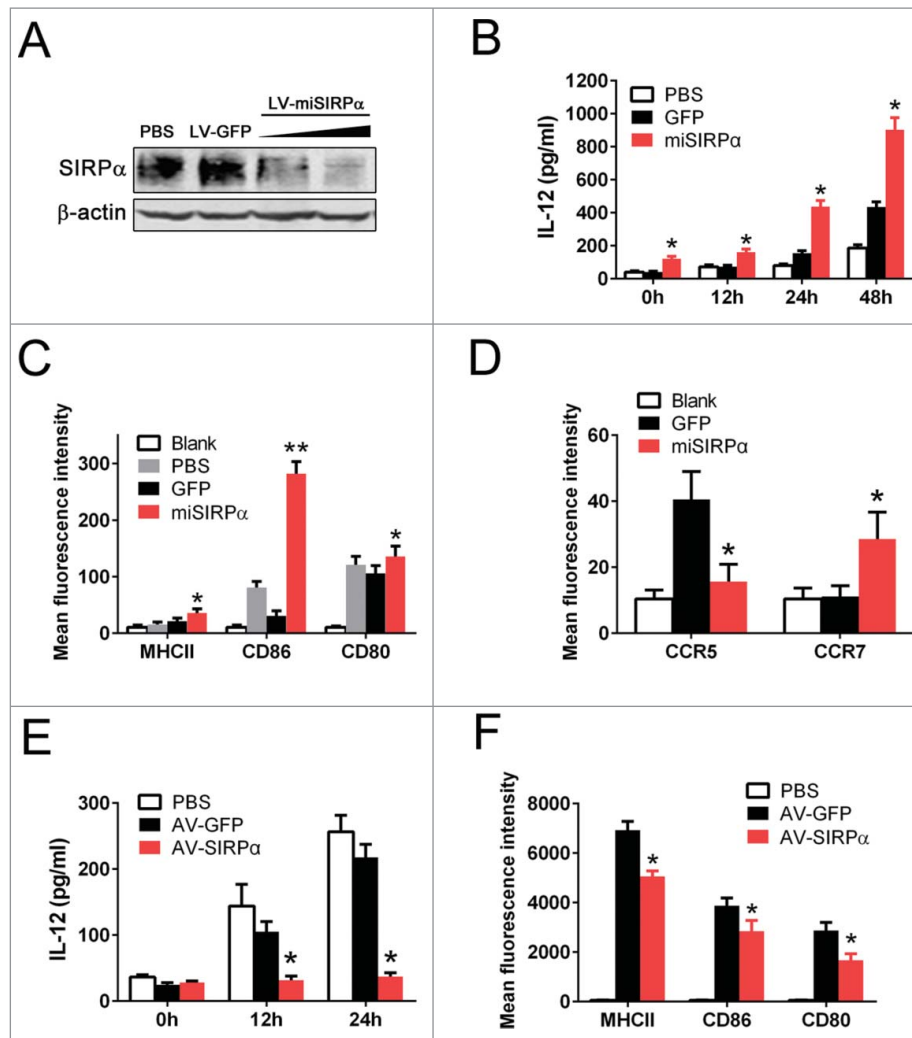


Figure 2. SIRP α controls the maturation and cytokine production of DCs. (A) Mouse BMDCs were infected with lentiviral vectors expressing miRNA targeting SIRP α or GFP control. The expression levels of SIRP α were analyzed 72 h after infection at an MOI of 50 or 80. (B) ELISA measurement of IL-12 production at the indicated times after lentiviral infection. Experiments were repeated three times yielding similar results. * $p < 0.05$ versus GFP controls. (C) FACS analysis of surface expression of costimulatory factors and MHC class II molecules on DCs 48 h after lentiviral infection. (D) FACS analysis of surface expression of CCR7 or CCR5 on DCs 48 h after lentiviral infection. Experiments were repeated three times yielding similar results. (E) ELISA measurement of IL-12 production at the indicated times after adenoviral infection. AV-GFP, adenoviral vector expressing GFP control; AV-SIRP α , adenoviral vector expressing exogenous SIRP α . (F) FACS analysis of surface expression of costimulatory factors and MHC class II molecules on DCs 48 h after adenoviral infection. Experiments were repeated three times with similar results.

than in autologous CD11c⁺ PBDCs. These results indicate that the expression level of SIRP α seems to be inversely linked to DC activation both *in vitro* and *in vivo* (Fig. 1C).

SIRP α controls the maturation and cytokine production of DCs

To elucidate the role of SIRP α in DC function, we generated a recombinant lentiviral vector expressing an interfering miRNA that effectively silences SIRP α expression (LV-miSIRP α) in mouse BMDCs (mDCs) (Fig. 2A). mDCs transduced with LV-miSIRP α spontaneously produced large amounts of IL-12 as compared with LV-GFP-transduced DCs or PBS controls (Fig. 2B). We also observed a higher expression of maturation markers, including CD80, CD86 and major histocompatibility complex (MHC) class II, in LV-miSIRP α -DCs or LV-shSIRP α -DCs (Fig. 2C, Fig. S1 and S2), suggesting an enhanced maturation in SIRP α -silenced DCs. One important outcome of the maturation process is that DCs acquire the capacity to home to lymph

nodes.²⁵ SIRP α silencing in DCs resulted in elevated expression of chemokine receptor CCR7 (Fig. 2D), which directs DCs to secondary lymphoid nodes.²⁶ In contrast, the chemokine receptor CCR5, which is thought to be involved in recruitment of immature DC to tissues,²⁷ had reduced expression in DCs transduced with LV-miSIRP α compared with GFP control. Consistent with that observation, mDCs expressing exogenous SIRP α (adenovirally directed overexpression of SIRP α , AV-SIRP α) showed considerably decreased IL-12 production and maturation marker expression (Fig. 2E and F). Taken together, these data suggest that SIRP α could negatively regulate DC activation and maturation.

SIRP α restrains the survival and homeostasis of DCs in lymphoid tissues

In addition to enhancing mDC maturation and cytokine production, silencing of SIRP α significantly prolonged DC lifespan after GM-CSF deprivation (Fig. 3A). By contrast, adenovirally directed overexpression of SIRP α (AV-SIRP α) significantly

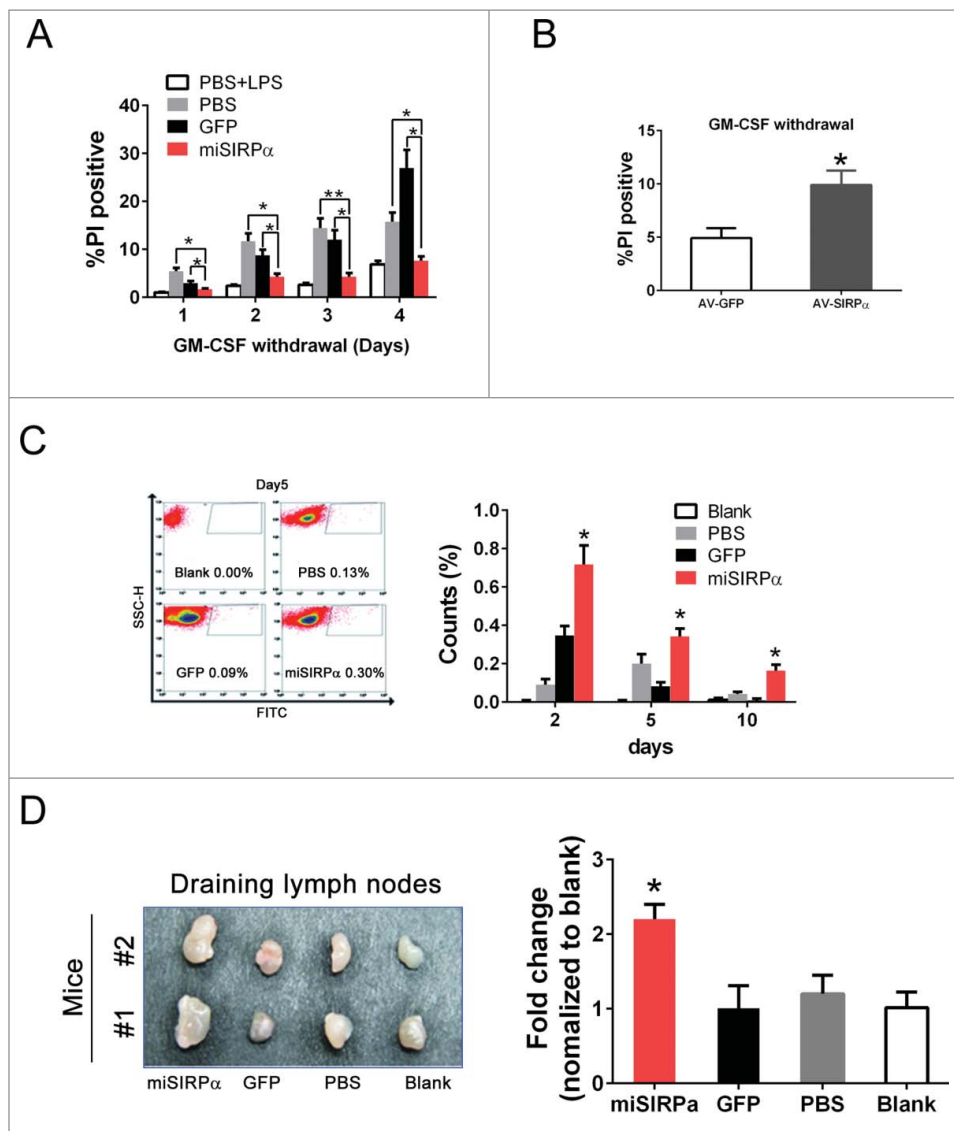


Figure 3. SIRP α restrains the survival and homeostasis of DCs in lymphoid tissues. (A & B) DC survival was negatively regulated by SIRP α . Viabilities of LV-miSIRP α -DCs, LV-GFP-DCs and other controls (A) or AV-SIRP α -DCs and AV-GFP control (B) were assayed by propidium iodide staining after 1–4 d of GM-CSF deprivation culture followed by analysis with flow cytometry. (C) BMDCs were either transduced with LV-miSIRP α or controls followed by LPS treatment. BMDCs were then stained with CFSE and injected into hind legs of syngeneic mice. Draining popliteal lymph nodes were harvested at the indicated times after DC injection, and PtdIns-negative populations were analyzed by flow cytometry. Experiments were repeated three times yielding similar results. (D) The secondary lymphoid organs were examined on day 10 after DC injection. Quantification of lymphoid organ volumes is shown in the right panel. * $p < 0.05$ vs. GFP controls.

reduced the survival of DCs relative to adenoviral GFP transduction (Fig. 3B), revealing an inhibitory function for SIRP α in DC survival. To determine whether SIRP α contributes to the survival and maturation of DCs *in vivo*, we delivered peptide-pulsed, CFSE-stained and LPS-treated miSIRP α -DCs or GFP-DCs subcutaneously to syngeneic mice. We found that the percentage of CFSE⁺ miSIRP α -DCs in draining popliteal lymph nodes was significantly higher than that of CFSE⁺ GFP-DCs (Fig. 3C). This disproportionate representation was sustained for at least 10 d after DC delivery. Moreover, the average volume of draining lymph nodes exposed to miSIRP α -DCs was enlarged at least 80–100% compared with that of control mice, indicating that LV-miSIRP α transduction enhanced the number and longevity of leukocytes resident in secondary lymphoid tissues (Fig. 3D). Collectively, these results indicate that the increased survival and activation of miSIRP α -DCs resulted in increases in the size, cellularity and functions of secondary lymphoid organs.

SIRP α -silent DCs prime enhanced T cell responses

The enhanced survival and activation exhibited by miSIRP α -DCs led us to hypothesize that they might also promote antigen-specific cytotoxic T lymphocyte (CTL) responses. Therefore, we evaluated the CD8⁺ OT-I T cell proliferative response to ovalbumin (OVA)-pulsed DCs by thymidine incorporation or CFSE labeling *in vitro*. As expected, silencing of SIRP α or anti-CD47 Ab blockage significantly enhanced the proliferation of CD8⁺ T cells relative to LV-GFP or PBS controls (Fig. 4A and B).

To further test whether the immunostimulatory potency of DCs is regulated by SIRP α *in vivo*, we transduced mDCs with LV-miSIRP α or LV-GFP lentiviral vectors, pulsed the transduced cells with OVA (Fig. 4C) or self-tumor associated antigen tyrosinase-related protein-2 (TRP-2) (Fig. 4D), matured the cells *ex vivo* with LPS and then subcutaneously injected them into syngeneic C57BL/6 mice. The functional status of

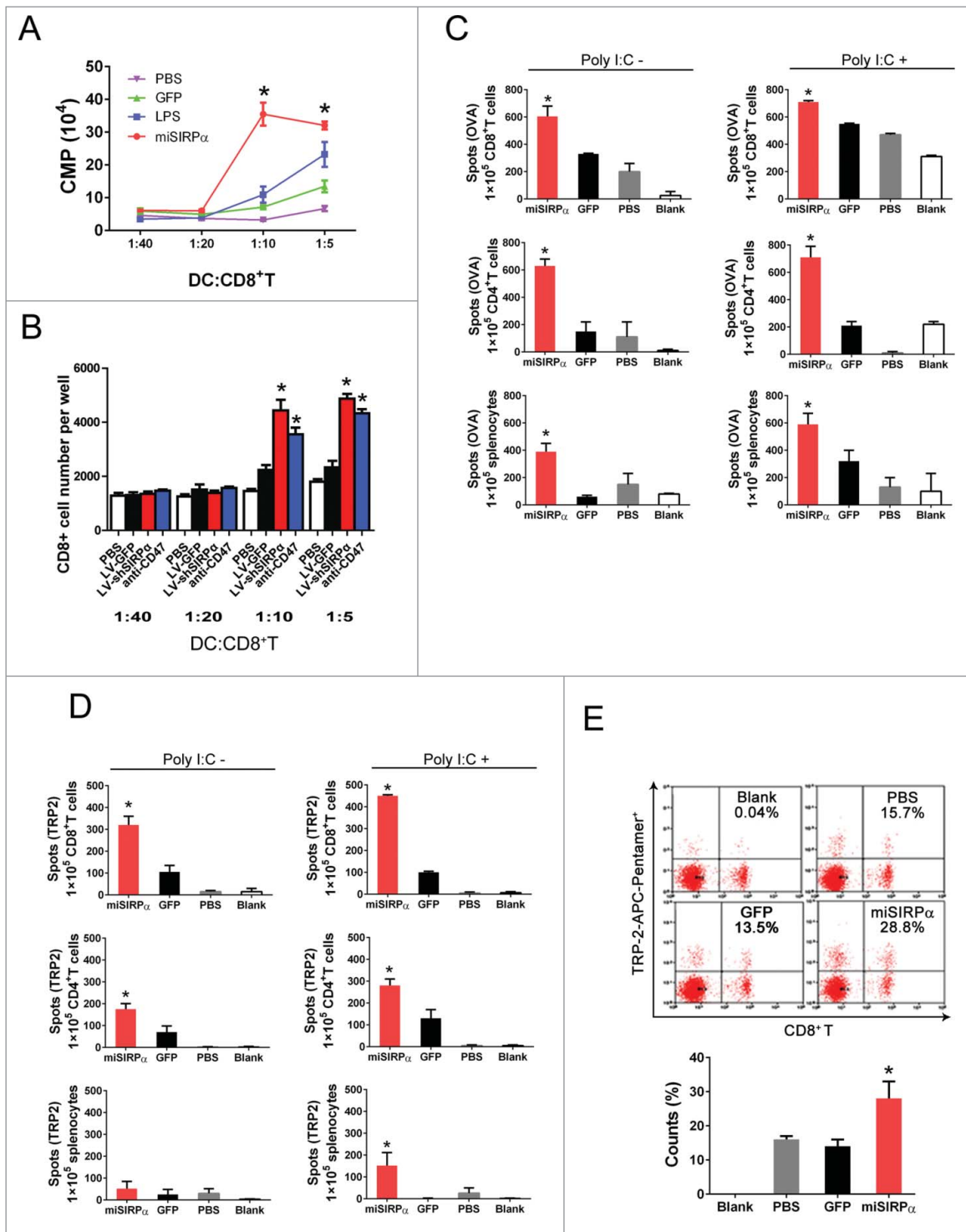


Figure 4. SIRP α -silent DCs prime enhanced T cell responses. (A & B) Stimulatory effect of LV-miSIRP α -DCs on CD8⁺ T cell proliferation *in vitro*. CD8⁺ OT-I-specific T cells isolated from the spleens of OT-I-transgenic mice were CFSE stained and co-cultured with irradiated DCs pulsed with H-2Kb OVA-I peptide in triplicate or CD47 antibody. [³H]TdR incorporation rates were then measured (A), and CFSE positive cells were counted microscopically (B); those shown are representative of three independent experiments. (C & D) IFN γ ELISPOT assays of splenocytes, CD4⁺ and CD8⁺ T cell responses to OVA (C) or TRP-2. (D) C57BL/6 mice were immunized with OVA or TRP2-pulsed (100 mg/mL), lentivirally transduced DCs *ex vivo* that had been matured with LPS, followed by no stimulation (left) or *in vivo* stimulation with poly:I:C (50 mg/mouse i.p.; right) daily for three consecutive days. Splenocytes pooled from immunized mice (two or three per group) or isolated CD8⁺ T cells or CD4⁺ T cells were subjected to IFN γ ELISPOT assays. Experiments were repeated three times with similar results. $p < 0.05$, miSIRP α -DCs compared with GFP-DCs. (E) Stimulatory effect of LV-miSIRP α -DCs on antigen-specific CD8⁺ T proliferation *in vivo*. Mice were immunized three times at 2-week intervals with LPS matured LV-miSIRP α -DCs or controls that had been pulsed with TRP2 *ex vivo*. Two months later, 1 week after a booster DC vaccine, data for TRP-2-APC-Pentamer⁺ and CD8⁺ T cells were collected from one of three independent experiments. * $p < 0.05$ versus GFP and PBS controls.

the T cells was evaluated using interferon- γ (IFN γ) enzyme-linked immunosorbent spot (ELISPOT) assays. Mice injected with miSIRP α -DCs had significantly greater numbers of

antigen-specific IFN γ ⁺CD4⁺, IFN γ ⁺CD8⁺ T cells and IFN γ ⁺ splenocytes than did mice injected with GFP-DCs. Moreover, *in vivo* ligand (polyI:C) stimulation more effectively boosted

antigen-specific CTL responses in miSIRP α -DC recipients than in GFP-DC recipients.

We next tested the ability of SIRP α -silent DCs to prime an antigen-specific response *in vivo* by directly immunizing mice with TRP-2-pulsed, transduced DCs with *ex vivo* maturation by LPS. Pentamer staining showed that 28.8% of total CD8⁺ T cells were positive for TRP-2-pentamer in mice immunized with LV-miSIRP α -DCs, compared with only 15.7% and 13.5% in mice immunized with LV-GFP-DCs or PBS-DCs, respectively (Fig. 4E). These data indicate that silencing of SIRP α likely reduces the threshold of DC responsiveness, resulting in a high magnitude of T cell responses.

SIRP α inhibits DC activation and survival through sequestration of p85

The PI3K/Akt pathway is a key regulator of DC lifespan and is required for DC maturation and survival.²⁸ As SIRP α has been shown to recruit and signal via p85 subunit of PI3K, we thus investigated whether PI3K mediated SIRP α regulation of DC function. As shown in Fig. 5A, endogenous SIRP α was constitutively bound to p85 in DCs. Silencing of SIRP α led to a significant activation of PI3K as indicated by *in vitro* PIP3 production (Fig. 5B) while overexpression of SIRP α inhibited LPS-induced Akt phosphorylation, which normally occurs as a consequent event of PI3K activation (Fig. 5C). These results indicate that SIRP α might compete with the p110 catalytic subunit to bind p85, thus inhibiting PI3K activity. Likewise, overexpression of a dominant negative mutant of Akt (AV-AKT-T308A/S473A) significantly reduced IL-12 production in DCs transduced with LV-miSIRP α relative to AV-GFP controls (Fig. 5D). We also tested the effects of selective inhibitors against PI3K (wortmannin, Ly294002) on LV-miSIRP α -mediated DC activation and survival. Inhibition of their activities abrogated LV-miSIRP α -mediated IL-12 production (Fig. 5E) and DC maturation (Fig. 5F), suggesting that the PI3K/Akt pathway is involved in LV-miSIRP α -mediated DC activation. PI3K inhibitors also substantially prevented the LV-miSIRP α -mediated rescue of DC death following GM-CSF deprivation (Fig. 5G). Furthermore, adenovirally directed overexpression of a dominant-negative mutant of Akt (AV-AKT-T308A/S473A) greatly reduced the survival of DCs transduced with LV-miSIRP α relative to AV-GFP control (Fig. 5H). These results suggest an essential role of the PI3K/Akt pathway in SIRP α regulation of DC survival and activation.

SIRP α silencing in DCs enhances antitumor immune effects

The observed regulatory role of SIRP α in DC activation prompted us to test whether SIRP α -silent DCs might induce potent antitumor immunity against subcutaneous E.G7-OVA lymphomas to correlate *in vitro* findings. Groups of 8 to 10 mice were primed by subcutaneous injection of 2×10^6 DCs that had been pulsed with OVA and matured *ex vivo* with LPS. One week after vaccination, animals were challenged by local injection of 1×10^6 (E.G7) tumor cells, and tumor growth or overall survival was assessed at regular intervals by a blinded observer to determine therapeutic efficacy. As expected, immunization with a single dose of OVA-pulsed miSIRP α -DCs

completely blocked E.G7 tumor growth in all mice tested, whereas inoculation with GFP-DCs or PBS-DCs failed to inhibit tumor growth in most animals (Fig. 6A). In addition, an IFN γ ELISPOT assay showed that vaccination with peptide-pulsed miSIRP α -DCs significantly increased the numbers of antigen-specific IFN γ ⁺CD8⁺ T cells compared to GFP-DCs and PBS-DCs (Fig. 6B). These data demonstrate that silencing SIRP α is an effective means of priming DCs for the prevention of primary autologous tumor outgrowth after local challenge.

To determine the efficacy of SIRP α -silent DCs for the treatment of established disease, we exploited the well-established E. G7-OVA lymphomas and poorly immunogenic B16 metastases models. Groups of 10 animals with established melanoma or metastases were inoculated weekly with DCs for 3 weeks. In both the pre-established E.G7 experiments and the B16 model, local tumor outgrowth was significantly inhibited by vaccination with SIRP α -silent DCs, whereas GFP-DC or PBS-DC vaccines failed to inhibit tumor growth in most animals (Fig. 6C and D). Furthermore, we observed that immunization of mature miSIRP α -DCs pulsed with TRP-2 peptide also significantly increased the numbers of antigen-specific IFN γ ⁺CD4⁺, IFN γ ⁺CD8⁺ T cells and IFN γ ⁺ splenocytes relative to vaccination with control GFP-DCs (Fig. 6E).

To further evaluate the role of CTLs in miSIRP α -DC-induced tumor protection, we inoculated 10 naive mice with B16 melanoma cells 7 d after injection with DCs. Splenocytes from miSIRP α -DC-treated mice or from control groups were then transferred to these mice 1 d before challenge with B16 melanoma cells. As expected, almost all of the mice that had received splenocytes from the miSIRP α -DC-treated mice were efficiently protected from tumor growth, whereas mice that had received control splenocytes were not (Fig. 6F). To determine the role of CD4⁺ and CD8⁺ T cells in tumor protection, we repeated this experiment using splenocytes depleted of CD4⁺ or CD8⁺ T cells from miSIRP α -DC-treated mice. One day after T cells infusion, these mice were injected with B16 melanoma cells. As shown in Fig. 5F, both CD4⁺ T and CD8⁺ T cells were required to mediate the antitumor effects.

Discussion

Here, we provide the evidence for an inhibitory function of SIRP α in DC survival and activation. The silencing of SIRP α resulted in a significant increase in the number of antigen-pulsed DCs in the draining LNs. The effect of SIRP α knockdown on DC survival *in vivo* suggests that it might not only increase the survival of DCs at the site of injection but also enhance the longevity of Ag-bearing DCs after migration to the LNs. SIRP α knockdown also led to an upregulation of CCR7, which favor the entry of DCs into afferent lymphatics and homing to the T cell areas.²⁶ These mechanisms might operate together to enhance the longevity of mature DCs in secondary lymphoid tissues and thereby increase adaptive immune responses. Interestingly, SIRP α has also been shown to be necessary for DC homeostasis in lymphoid tissues. The number of CD11c⁺ DCs was reduced in secondary lymphoid tissues of mice expressing a mutant form of SIRP α that lacks the cytoplasmic region.²⁹ It is of note that this mutant SIRP α is structurally similar to SIRP γ , another member of the SIRP family

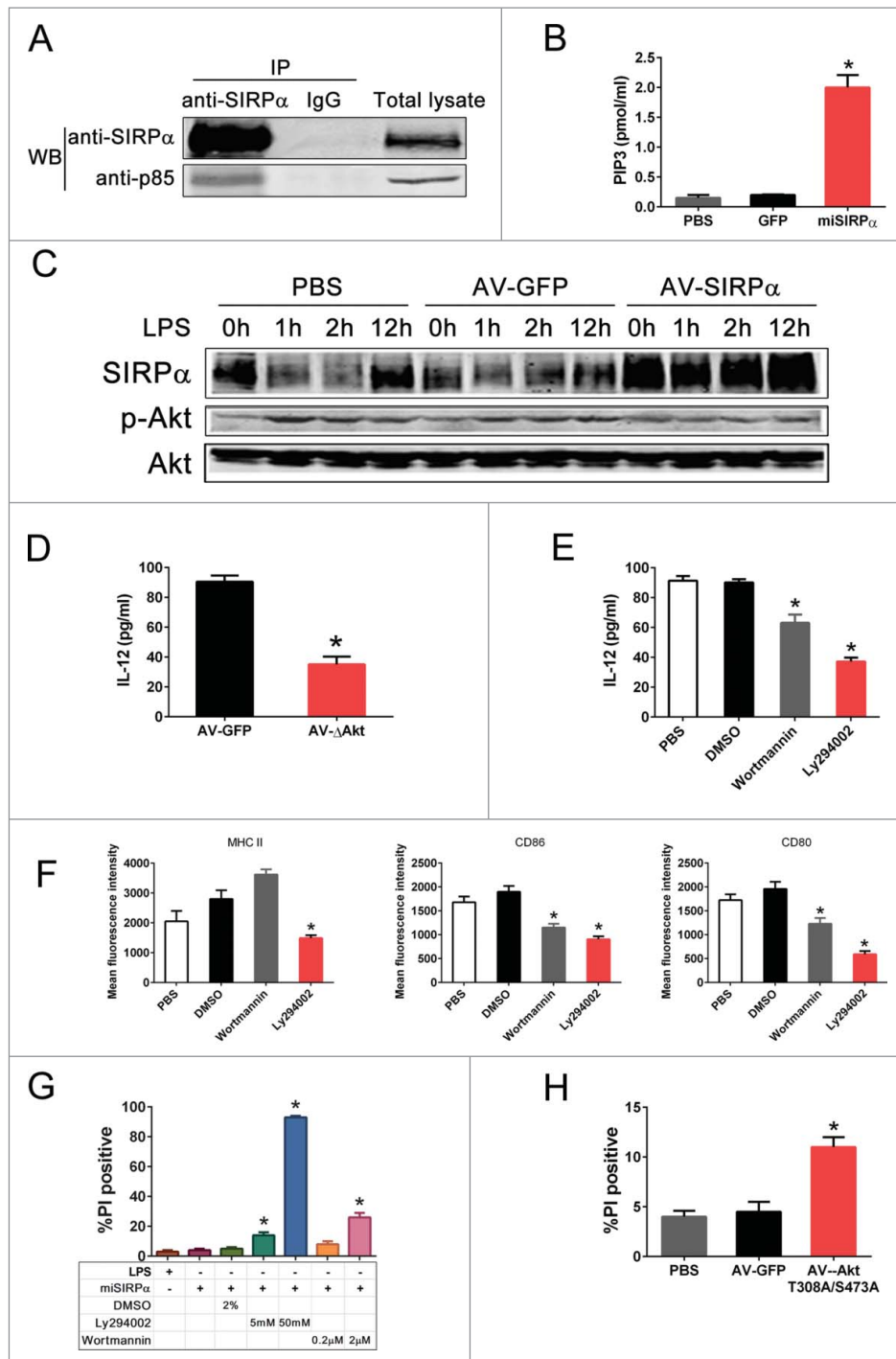


Figure 5. SIRT α inhibits DC activation and survival through sequestration of p85. (A) BMDC lysates were immunoprecipitated for endogenous SIRT α and immunoprecipitates were probed with anti-SIRT α and anti-p85 antibodies. (B) PI3-Kinase activity was negatively regulated by SIRT α . BMDC lysates were collected 48 h after infection with LV-miSIRT α , LV-GFP or PBS control and assayed for PI3-Kinase activity by ELISA. (C) BMDCs were infected with adenoviral vectors (AV-GFP and AV-SIRT α) for 24 h and the level of total and phospho-Akt were ascertained by Western blotting after incubation with 100 ng/mL LPS for the indicated times. (D) Overexpression of dominant-negative Akt prevented the LV-miSIRT α -mediated IL-12 production by DCs. After infection with LV-miSIRT α for 24 h, BMDCs were infected with AV-AKT-T308A/S473A or the controls for 48 h, and the amounts of IL-12 secreted were measured by ELISA. The experiments were repeated three times yielding similar results. (E & F) Effects of inhibitors of MAPK or PI3K pathway on LV-miSIRT α -mediated IL-12 production and DC maturation. After infection with LV-miSIRT α for 24 h, BMDCs were incubated with the indicated concentrations of PI3K inhibitors or DMSO control for 24 h. ELISA measurement of IL-12 secreted by DCs and FACS analysis of surface expression of costimulatory and MHC class II molecules are shown from one of three independent experiments. * $p < 0.05$ vs. DMSO controls. (G) In the presence of PI3K-Akt pathway inhibitors, knockdown of SIRT α expression failed to enhance DC survival. After infection with LV-miSIRT α or incubation with 100ng/mL LPS for 24 h, BMDCs were treated with the indicated concentrations of PI3K inhibitor Ly294002, Wortmannin or DMSO control. Cell viability was assessed 2 d later using PI staining. Experiments were repeated three times yielding similar results. (H) LV-miSIRT α -transduced BMDCs were further infected with adenoviral vectors expressing dominant-negative mutant of Akt (AV-AKT-T308A/S473A) or GFP control. The survival of DCs was examined as described above. * $p < 0.05$ versus GFP and PBS controls. Experiments were repeated three times with similar results.

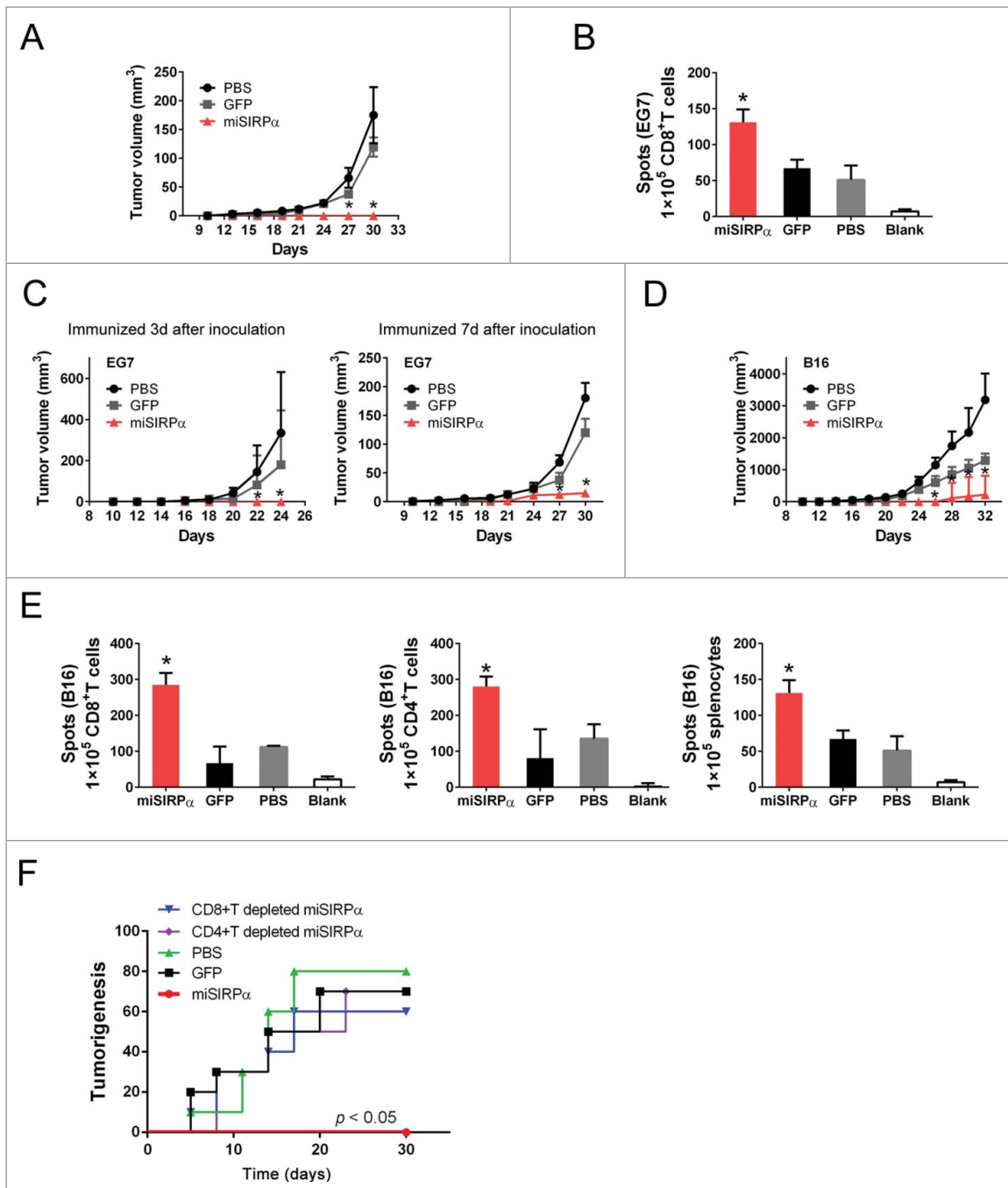


Figure 6. SIRP α silencing in DCs enhances antitumor immune effects. (A & B) Antitumor immunosurveillance activity against EG7. Syngeneic C57BL/6 mice were vaccinated with 2×10^6 LV-miSIRP α -DCs or controls pulsed with OVA peptide 7 d before challenge with 1×10^6 OVA positive EG7 cells. Tumor sizes were measured (A) and CD8⁺ responses to OVA were assessed by IFN γ ELISPOT assay (B). Tumor growth curves ($n = 6$ mice per group) represent one of two repeated experiments. $p < 0.01$, LV-miSIRP α -DCs compared with GFP-DCs. (C & D). Antitumor activity against pre-established or established tumors. C57BL/6 mice were inoculated subcutaneously with 1×10^6 EG7-OVA or 1×10^5 B16 tumor cells and 3 d or 7 d later were immunized with 2×10^6 OVA or TRP2-pulsed LV-miSIRP α -DCs or control DCs *ex vivo* matured with LPS twice at a weekly interval, followed by *in vivo* poly:I:C stimulation i.p. after each DC immunization. Tumor growth curves ($n = 6$ mice per group) represent one of two repeated experiments. $p < 0.01$, LV-miSIRP α -DCs compared with GFP-DCs. (E) IFN γ ELISPOT assays of CD8⁺ T cell, CD4⁺ T cell or splenocytes responses to TRP-2 were recorded. $*p < 0.01$ vs. GFP controls. (F) Characterization of the antitumor immune response induced by LV-miSIRP α -DCs. 1×10^7 pooled splenocytes or 1×10^7 CD8⁺ or CD4⁺ T cells isolated from the pooled splenocytes (two or three per group) of different groups of mice were transferred to host mice 1 d before B16 melanoma cell inoculation. Tumorigenesis was monitored for 30 d. Shown are the pooled results of two independent experiments ($n = 10$).

which is functionally involved in T-cell proliferation and activation.³⁰ As the mutant SIRP α is still able to ligate CD47 on T cells, it is not yet known if this mutant can represent a functionally deficient SIRP α in DCs.

In addition to restraining the survival of DCs, SIRP α also controls the activation and output of DCs. Activation of DCs occurs in two phases: maturation and licensing. We found that

SIRP α -silent DCs undergo a maturation process even in the absence of inflammatory stimuli, such as LPS. Furthermore, DCs with SIRP α knockdown acquired an enhanced capacity to home to lymph nodes, one important consequence of the maturation process. On the other hand, miSIRP α -DCs spontaneously produced large amounts of IL-12, a licensing cytokine that mediates the polarization of activated CD4⁺ T cells to a

Th1 phenotype such that they aid in eliciting potent CD8⁺ CTL responses.³ Thus, by prolonging DC lifespans, inducing optimal maturation and DC activation, accompanied by IL-12 production, silencing of SIRP α could, as was expected, lead to much greater DC-mediated T cell proliferation and IFN γ production. These results suggest that SIRP α -silent DCs might elicit CTL responses via at least two sequential mechanisms: the enhanced DC maturation facilitates initiation of CTL responses and the increased IL-12 production intensifies the magnitude of CTL responses. Moreover, SIRP α -silent DCs might be capable of turning off regulatory T cells by enhancing DC maturation and cytokine production. A recent finding indicates that a thymic SIRP α ⁺ but not SIRP α ⁻ conventional DC subset can support the development of central tolerance against circulating peripheral Ags (CCR2) and induce CD4⁺CD25⁺ Foxp3⁺ T reg cells both *in vitro* and *in vivo*.³¹

Signaling events downstream of SIRP α and their functional impact on DC maturation and activation are incompletely understood. In DCs, TLR signaling induced by inflammatory stimuli has been shown to activate the PI3K/Akt pathway to promote DC longevity.³² We show that activation of TLR signaling also induces a transient downregulation of SIRP α and a subsequent restoration of its expression. More importantly, we find that SIRP α is constitutively associated with the p85 subunit of PI3K in DC, which might interfere with activation of the p85/p110 PI3K heterodimer. Indeed, as seen in the PI3K kinase assay, the silencing of SIRP α resulted in a significant increase in PI3K activity, whereas overexpression of SIRP α inhibited PI3K activation. Consistent with the known role of the PI3K/Akt pathway in DC survival, SIRP α -silent DCs failed to exhibit a prolonged lifespan when treated with specific small inhibitors of the PI3K/Akt pathway or when expressing the dominant-negative form of Akt. It was previously demonstrated that Akt deficiency results in defective DC maturation and activation.^{28,33} In light of the dependence of Akt on PI3K, that finding agrees with our results showing that the recruitment of the PI3K p85 subunit by SIRP α impaired activation of the PI3K/Akt pathway, thus endowing DCs with tolerogenic properties. These results provide strong evidence for a key role of SIRP α in regulating Akt-dependent DC survival and maturation. Our data also suggest that the disappearance and reappearance of SIRP α during DC maturation might reflect an intrinsic process that is inversely linked to DC lifespan and plays a key role in controlling immune responses.

The efficacy of the DC-based tumor vaccine we tested was significantly improved by silencing SIRP α . This was reflected in the substantially retarded tumor growth associated with supra-physiological T cell expansion in C57BL/6 mice bearing E.G7-OVA lymphoma or highly aggressive and poorly immunogenic B16 melanoma cells. Silencing of SIRP α resulted in more robust and extended activation of DCs and achieved greater T cell proliferation, IFN γ production and cytolytic activity than was observed when DCs were treated with LPS. SIRP α -silent DCs also efficiently induced long-lived CD8⁺ memory responses without detectable signs of autoimmunity. The superior and unique immunostimulatory effect of miSIRP α -DCs may be due to

the broad roles of SIRP α in negatively regulating many proinflammatory signal transduction pathways, as indicated by the higher surface expression of costimulatory molecules, the more selective but increased production of licensing cytokines, and the enhanced capacity to initiate antitumor immunity.³⁴⁻³⁶ Taken together, our study uncovers a critical role for SIRP α in restricting the DC-mediated adaptive immune response. The silencing of SIRP α in DCs might tip the balance from immune suppression to antitumor immunity as a collective outcome of the cells' effects on interactive networks of pro- and anti-inflammatory factors and cells in tumor-bearing mice. SIRP α inhibition might therefore be clinically useful for augmenting anti-infection and anticancer immunity.

Materials and methods

Antibodies and reagents

The following antibodies were used: anti-MHC class II, anti-CD80, anti-CD86, anti-CCR7 and anti-CCR5, anti-SIRP α and anti-CD11c (eBioscience, San Diego, CA); antibodies specific for AKT, Myc-tag, p85 (Cell Signaling Technology). Rabbit polyclonal SIRP α antibody was generated in our laboratory against residues in the cytoplasmic domain.³⁷ LPS and OVA were from Sigma-Aldrich. The CpG oligodeoxynucleotide 1826 was purchased from Invivogen. The PI3-Kinase inhibitors Ly294002 and wortmannin were purchased from Calbiochem. CFSE was purchased from Molecular Probes. DC viability was assessed using propidium iodide (PI; Sigma). TRP2 peptide (VYDFVWL) was purchased from SANGON.

Animals and cell lines

Male C57BL/6 mice (6–8 weeks) were purchased from Shanghai Experimental Center (Chinese Science Academy) and maintained in the barrier facility under pathogen-free conditions. OT-1 TCR transgenic mice were purchased from Jackson laboratory. B16 melanoma cells and OVA-expressing EL4 (EG7) cells were grown in 1640 medium (Gibco BRL, Gaithersburg, MD), containing 10% FBS. 293T and 293A cells were maintained in DMEM supplemented with 10% FBS.

Human PBMCs and tumor samples

The peripheral blood cells and tumor tissues were obtained from healthy volunteers or patients in Eastern Hepatobiliary Surgery Hospital. All procedures were performed according to investigator's protocols approved by the Second Military Medical University Research Ethics Committee.

Lentiviral vector production

The lentiviral vectors used in this study were constructed using the BLOCK-iTTM lentiviral Pol II miR RNAi expression kits (Invitrogen). Predesigned miRNA targeted against SIRP α was provided by Invitrogen, with the following sequences: miSIRP α : 5'-TGCTGTCT ATGAGCAGATGAGTTCACGTTTTG GCCACTGACTGACGTGAACTCCTGCTC ATAGA - 3'.

Transduction of bone marrow-derived DC with lentiviral vectors

Mouse BMDCs were prepared as described previously. Lentiviral infections of BMDCs were performed on six-well plates. After 5 d of culture in mGM-CSF (10 ng/mL) and mIL-4 (1 ng/mL, PeproTech), immature DCs were removed to serum-free 1640. The transductions were conducted by adding concentrated supernatant at an MOI of 50 or 80 in the presence of 8 μ g/mL polybrene(Sigma).

Cytokine and PI3-Kinase activity assays

Cytokine or PIP3 levels in culture-derived supernatants were determined using commercial ELISA kits for IL-12 (eBioscience). For PI3-Kinase Activity (Echelon Biosciences Inc.), BMDC lysates were collected 48 h after infection with LV-miSIRP α , LV-GFP or PBS control. PI3 kinase was immunoprecipitated with 5 μ L of rabbit antibody against PI3 kinase (which coprecipitates the p85 subunit of PI3 kinase) and 60 μ L of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.). PI3 kinase activity in the immunoprecipitates was analyzed with PI3 kinase enzyme-linked immunosorbent assay (ELISA) (from Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's instructions.

Western blotting analysis

Samples of cell lysates were separated by 8–12% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Duplicate membranes were probed with polyclonal or monoclonal antibodies (mAbs) such as anti-SIRP α , anti-Akt or Akt, and then incubated with anti-mouse or rabbit HRP (Santa Cruz, CA). Specific bands were detected using an IRDye 800CW-conjugated secondary antibody and LI-COR imaging system (LI-COR Biosciences).

Flow cytometry analysis

1×10^5 cells were suspended in PBS containing 1% BSA and were stained with various fluorochrome-conjugated mAbs for 30 min on ice. Flow cytometry analysis was performed using Moflo-XDP (Beckman Coulter, Fullerton, CA) equipped with Summit 5.1 Software. The frequency of TRP-2-specific CD8⁺ T cells was determined by staining lymph node cells with TRP-2-APC-pentamer (Pro-Immune, Bradenton, FL, USA). Briefly, We stained cells with anti-CD8⁺-FITC (clone 53-6.7; BD Biosciences) and APC-H-2Kb/TRP-2 (SVYDFFVWL, SVL) Pro5 pentamer (ProImmune) for 20 min at room temperature and then lysed erythrocytes with red blood cell lysing buffer (Sigma-Aldrich) for 7 min. TRP-2-specific CD8⁺ T cells were then determined by analysis by flow cytometry.

Proliferation of CD8⁺ T cells

Spleens of OT-1 were collected, and CD8⁺ T lymphocytes were enriched by immunomagnetic kit following the manufacturer's protocol (StemCell Technologies, Inc.) and seeded onto 96-well plates at 2×10^5 cells/well. Bone marrow DCs derived from 8-d

culture of lentivirus-transduced or control groups were irradiated at 3 Gy and seeded onto the 96-well plates with T cells in triplicates at the stimulator (DC)-to-effector (T cells) ratios shown in Fig. 4A. Three days later, the wells were pulsed with 1 μ Ci/well [³H]thymidine and harvested 18–20 h later with a Packard Micro-mate cell harvester. The ³H incorporation was determined as counts per minute through a Packard Matrix 96 direct β counter.

IFN γ ELISPOT assay

C57BL/6 mice were immunized with OVA-pulsed or TRP2-pulsed DCs (100 μ g/mL) that had been transduced with lentiviral vectors and matured with LPS *ex vivo*, and then stimulated *in vivo* with polyI:C (50 μ g/mouse i.p.) daily for three consecutive days. Splenocytes pooled from immunized mice (two or three per group) and single-cell suspensions were obtained by crushing and passing through nylon mesh. CD8⁺ or CD4⁺ T cells were isolated by positive selection using a CD8⁺ or CD4⁺ T cell isolation kit (StemCell Technologies, Inc.). IFN γ ELISPOT assays were conducted according to the manufacturer's protocol (Quick SpotTM IFN γ ELISPOT kit, Dakewe).

DC immunization and tumor models

E.G7-OVA lymphoma, B16 melanoma cells (ATCC) were expanded in culture and injected subcutaneously into the right flanks of C57BL/6 mice (1×10^6 E.G7-OVA cells/mouse or 1×10^5 B16 melanoma cells/mouse). For prophylactic or therapeutic DC vaccines, miSIRP α -DCs or control DCs were pulsed with OVA proteins (100 μ g/mL) for E.G7-OVA cells or TRP-2 peptide (10 mg/mL) for B16 or B16F10 cells and injected subcutaneously via footpads (2×10^6 DCs/mouse). Prophylactic DC vaccine treatment was done 7 d before tumor injection or therapeutic DC vaccination was done 3 d after tumor injection with administration of polyI:C (50 μ g/mouse) in 7, 14 and 21 d after tumor injection. Tumor volumes were recorded every 2 d.

Statistical analysis

All values are expressed as the mean \pm SEM of data obtained from at least three experiments. Comparisons between groups were made with the Student's *t* test and ANOVA test. A significance level of $p < 0.05$ was used.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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