

PD-1/PD-L1 axis suppressed homeostatic proliferation–driven antitumor immunity during recovery from lymphopenic condition.

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Research

Keywords: PD-1/PD-L1, lymphopenic condition, immunotherapy, melanoma, homeostatic proliferation.

Posted Date: May 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-25668/v1>

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Abstract

Background Lymphopenic condition after radiotherapy or chemotherapy might be able to create an environment to mount an efficient antitumor immunity through the homeostatic proliferation of residual or adoptive lymphocytes. However, the antitumor immunity seems to decrease rapidly with the growth of tumor, and the mechanism remain elusive.

Methods In this study, we first examined the dynamic change of PD-1 expression on T cells and analyzed the connection between PD-1 expression and the exhaustion of CTLs when the number of T cells recovered from lymphopenic conditions. Anti-PD-1/PD-L1 therapies, including anti-PD-1 antibodies, PD-1 or PD-L1 knockout by Crispr-Cas9 system, were used in the study to evaluate the role of PD-1/PD-L1 block on the antigen recognition, differentiation, activation, killing, survival and prognosis of HP cells.

Results Our study found that in the mouse model of homeostatic proliferation, although T cells proliferated continuously in lymphopenic host, the number of IFN- γ -releasing CD8⁺T cells rapidly decreased in course of homeostatic proliferation, indicating the immunosuppressive state in tumor microenvironment. The expression of PD-1 on T cells, increased gradually in course of homeostatic proliferation, were significantly negatively related to the cytotoxicity of effector T cell. Blocking of PD-1/PD-L1 axis by PD-1 antibody promoted HP cells to recognize tumor associated antigen (TAAs) and resulted in the activation of DC cells. It can also enhanced the number of IFN- γ -releasing CD8⁺ T cells and the cytotoxicity of effector T cell in lymphopenic mice. Furthermore, PD-1 antibody induced a portion of tumor-specific CTL convert to central memory T cells (TCM). Finally, we found that Crispr/Cas9-mediated knockout of PD-1 in T cells or PD-L1 in melanoma cells significantly enhanced homeostatic proliferation-driven antitumor responses and inhibited tumor growth.

Conclusion These findings suggested PD-1/PD-L1 played an important role in the formation of immune tolerance during the period of homeostatic proliferation. Anti-PD-1/PD-L1 therapies may be used to enhance antitumor immunity during recovery from lymphopenic condition after chemotherapy or radiotherapy.

Introduction

Under normal circumstances, the immune system can recognize and remove tumor cells in the tumor microenvironment. However, tumor cells adopted different strategies to inhibit the immune system of human body and cannot be killed by immune cells, so that they can survive in all stages of anti-tumor immune response. The purpose of cancer immunotherapy is to induce and maintain antitumor immune response. That is an in vivo generation of a large number of functional tumor antigen-specific T cells that are not restrained by normal or cancer-induced tolerance mechanisms.[1].

T cell homeostasis refers to the relative constant number of T cell populations at the overall level. If T cells were significantly reduced for some reason, the remaining T cells in the periphery will proliferate spontaneously to restore the normal size of T cell population and maintain homeostasis [2]. In the

condition of sharp decrease of T cells, T cells proliferate steadily through the action of self-peptide-MHC complex presented by dendritic cells to restore the original T-cell pool size. The proliferated T cells have remarkable self-responsiveness and function of both effector and memory T cells [3]. Tumor associated antigen (TAAs) are usually poor target when lymphocytes are sufficient, because they are often self-antigens and have weak immunogenicity. lymphopenic conditions can create an environment to induce an enhanced antitumor immunity through the homeostatic proliferation of residual or adoptive lymphocytes as shown in lymphopenic animal models[4, 5]. However, the antitumor immunity seems to decrease rapidly with the growth of tumor in the later stage of homeostatic proliferation, and the mechanism remain elusive [6].

The PD-1 is a key regulator of T cell function and fate. It was up-regulated after T cell activation, but was considered to be a sign of T cell exhaustion, which is a kind of cell state with low function found in tumor infiltrating lymphocytes of patients with chronic virus infection or advanced cancer. [7, 8]. It is worth noting that the expression of PD-1 ligand PD-L1 and PD-L2 in a variety of tumors is related to poor prognosis. The anti-PD-1/PD-L1 blocking antibody has shown effective anti-tumor immune response and promising results in a variety of cancer patients [9-11].

PD-1/PD-L1 axis is not only involved in tumor immune escape, but also closely related to chronic infection, transplant rejection and autoimmune diseases[12, 13]. However, the role of PD-1/PD-L1 axis on antitumor immunity induced by homeostatic proliferation of T cells in lymphopenic conditions remains elusive. In this study, we first examined the dynamic change of PD-1 expression on T cells and analyzed the connection between PD-1 expression and the exhaustion of CTLs when the number of T cells recovered from lymphopenic conditions. Anti-PD-1/PD-L1 therapies, including anti-PD-1 antibodies, PD-1 or PD-L1 knockout by Crispr-Cas9 system, were used in the study to evaluate the effects of PD-1/PD-L1 block on homeostatic proliferation-driven antitumor immunity. We further analyzed the role and mechanism of PD1/PD-L1 signaling on the antigen recognition, differentiation, activation, killing, survival and prognosis of homeostasis proliferating T cells. This study is not only important for elucidating the mechanism of the wane of homeostatic proliferation-driven antitumor responses, but also provides a scientific basis for developing novel combined treatment of anti-PD-1/PD-L1 therapy during recovery from lymphopenic condition after chemotherapy or radiotherapy.

Materials And Methods

Animals and transplantation. 6-8 weeks old female BALB/c or C57BL/6 mice were purchased from Laboratory Animal Center of Southern Medical University (Guangzhou, China), and were housed under sterilized conditions. Animal studies were carried out according to the institutional guidelines and approved by the Animal Care and Use Committee of Southern Medical University, China. C57BL/6 mice received a sub-lethal (650cGy) or lethal (900cGy) dose of total body irradiation on the day before transplantation. The irradiated mice received intravenous infusion of 2×10^6 splenic T cells and 5×10^6 of T cell-depleted marrow cells, respectively.

Tumor challenge and measurement. B16 melanoma cells were resuspended in PBS at a concentration of 5×10^6 cells/mL. Each mouse was injected s.c. laterally with a volume of 0.1ml (5×10^5 tumor cells). The size of the tumor was measured by a caliper, and the average value was determined by taking the maximum diameter of the tumor and its perpendicularity. Euthanasia was carried out in mice with tumor length greater than 20 mm.

ELISpot assays. IFN- γ ELISpot kits (BD Biosciences) were used according to the manufacturer's instructions. Briefly, 30Gy-irradiated B16 or 4T1 (5×10^4) cells and splenocytes (1×10^5) were cocultured in 96-well plates precoated with mouse IFN- γ (BD Biosciences) at 37°C for 20h in complete RPMI medium in triplicate. After the cell suspension was rinsed with deionized water, biotinylated anti mouse IFN - γ antibody (2 μ g / ml) was added and incubated at room temperature for 2 hours. After extensive washing, streptavidin horseradish peroxidase solution was added and incubated at room temperature for 1 hour. After washing, an aminoethyl carbazole substrate solution was added and incubated for 15 min. Spots were counted under a stereomicroscope after washing the plate.

In vivo depletion of T and NK cells. To deplete specific immune effector cell subsets before and during chemotherapy or radiotherapy, the transplanted mice received intravenous injection of 0.3 mg monoclonal antibody of anti-CD4⁺ (clone GK1.5, rat IgG2b) or anti CD8⁺ (clone Lyt-2.1, mouse IgG2b) or 0.5 mg of antiasialo GM1 antibody (Wako Pure Chemical Industries, Ltd). The intravenous injection started on the inoculation day of 4T1 or B16 cells, and was repeated every 5 to 6 days throughout the experiment to ensure the depletion of target cell types.

CFSE labeling. Purified T cells were resuspended in cold PBS at a concentration of 2×10^7 cells/mL. An equal volume of PBS containing 5 mmol/L CFSE was added, and cells were incubated at room temperature for 6 min. The cells were washed with an excess of cold FCS and then washed three times with a cold DMEM containing 10% FCS. As mentioned above, CFSE labeled T cells were injected intravenously and flow cytometry was used to analyze spleen cells or lymph node cells at a specified time point.

Crispr/Cas9-mediated knockout of PD-L1 on B16 melanoma cells.

Lentivirus containing Cas9 gene and PD-L1 sgRNA were constructed by Gene Technology Company (Genechem, Shanghai, China). B16 cells were plated at a density of 20~30% in a good condition on the day before infection,. The Cas9 lentivirus (containing puromycin resistance gene) was treated the B16 cells to construct a Cas9 overexpressing cell line. After puromycin resistant screening, the residual cells were treated the second PD-L1 sgRNA lentivirus (containing green fluorescent protein) to knockout the PD-L1 gene. The culture medium was changed to normal medium 12 hours after infection. Three days later, the cells were subjected to fluorescence activated cell sorting (FACS) with flow cytometer for further experiments.

Crispr/Cas9-mediated knockout of PD-1 on T cells.

Splenic lymphocytes were isolated from splenic cell suspension using density gradient centrifugation (Ficoll-Hypaque, TBD Science, Tianjin, China). CD3⁺ T lymphocytes were purified using mouse Pan T cell Isolation Kit(130-095-130, Miltenyi Biotec, Germany) followed by magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec, Germany). For electroporation, T cells were purified using negative selection to avoid the increase of cell mortality and cell activation.

For electroporation, cells were transfected with the Cas9-sgRNA RNPs by Amaxa™ 4D-Nucleofector (Lonza) using the Amaxa mouse T cells Nucleofector Kit (V4XP-3012, Lonza). 1×10⁶ purified T cells were washed with PBS, centrifuged with 300g for 5min and suspended in 100 μl transfection buffer and then transferred into electroporation cuvette. Program DN-100 was selected for transfection with high efficiency. After electroporation, cells were re-suspended in 500mL pre-warmed AIM-V medium containing 10% FBS and transferred into six-well cell plate and incubated at 37°C in 5% CO₂. The transfection efficiency was evaluated by fluorescent counts 24 h after electroporation. The knockout efficiency was evaluated by T7EN1 cleavage assay on day 7.

Results

Growth suppression of malignant melanoma in lymphopenic hosts is depended on CD8⁺ T cells from donor mice.

We first examined whether homeostatic proliferation of T cells could induce antitumor immunity against melanoma in lymphopenic murine mode. On the day of lethal (900cGy) or sub-lethal (650cGy) irradiation, C57BL/6 mice were injected s.c. with B16 melanoma cells shortly after the irradiation, and then bone marrow cells or T cells (without marrow cells) were infused respectively[14] (Figure 1A). The growth of tumors was substantially suppressed in the syngeneic BMT mice. Although the immunocompetent mice receiving lymphocyte infusion showed some antitumor effect at day 14 after the tumor inoculation, the tumor grew rapidly and caught up with the tumor growth of the control mice at day 23. The infusion of T cells suppressed tumor growth in sub-lethally irradiated mice also (Figure 1A). The similar effects were observed on lymphopenic mice induced by cyclophosphamide (CTX) (Figure 1B), suggesting that lymphopenia-induced homeostatic proliferation of T cells had antitumor effects on melanoma.

In the clinical adoptive T-cell therapy, the grafts were prepared from tumor-bearing patients. In order to observe the effect of tumor loading on the antitumor effect of adoptive T cell therapy, the mice bearing tumor for 16-18 days were selected as the donors of lymphocytes. The growth of tumors was significantly inhibited in the mice that received adoptive T-cell infusion from a tumor-bearing donor compared with a naive donor (Figure 1C), suggesting that some fraction of donor lymphocytes from tumor-bearing mice are primed in response to TAAs and remain responsive upon T-cell infusion.

To determine which lymphocyte subsets were responsible for the transfer of the antitumor immunity, depletion experiments of lymphocyte subsets were carried out on splenocytes derived from mice immunized against B16 cells before their adoptive transfer into irradiated B16 tumor-bearing mice. As

shown in Figure 1D, the antitumor effect of sublethal irradiation combined with infusion of CD8⁺ T-cell-depleted splenocyte suspension was significantly reduced with respect to standard sublethal irradiation and complete lymphocyte infusion (Figure 1D). However, the compromised effect of CD4⁺ T cell or NK cell depletion was less important than CD8⁺ T cells in proliferation-driven antitumor immunity (Figure 1D). These data suggest that CD8⁺ T cells are indeed the major lymphocyte subsets responsible for the transfer of the antitumor immunity.

Effects of sublethal radiotherapy on homeostatic proliferation of T cells.

To investigate the effect of combined treatment of sublethal irradiation and immune cell infusion on local anti-tumor immunity and system immunity, we analyzed the immune status in the tumor microenvironment and spleen after combined treatment in C57BL/6 mice. Flow cytometry analysis showed a significant increase of the percentage of CD3⁺CD8⁺ and CD3⁺CD4⁺ T cells in tumor-DLN and spleen in combined treatment mice (Figure 2A & 2B). To investigate whether the increased number of transferred splenocytes recovered in the spleen and lymph nodes of lymphopenic mice were due to cell proliferation, transferred splenocytes were labeled with CFSE and analyzed for CFSE dilution by flow cytometry in lymphopenic hosts. As shown in Figure 2C & 2D, transferred CD4⁺ and CD8⁺ T cells underwent several rounds of proliferation in tumor-draining lymph nodes and spleen on day 10 after infusion into sublethally irradiated mice (Figure 2C & 2D). These data suggest that combined treatment of sublethal irradiation and immune cell infusion can effectively enhance local anti-tumor immunity while expand system immunity.

Despite ongoing T cell proliferation in tumor-DLN of lymphopenic mice, functional tumor antigen-specific CD8⁺ T cells wane over time.

In an attempt to understand the relationship between antitumor immunity and the homeostatic proliferation of T cells, tumor-draining and non-draining lymph node were harvested on days 7, 14, and 21 and analyzed for CFSE dilution by flow cytometry. On day 7, there was about 28% CFSE dilution of T cells in the tumor-DLNs, indicating spontaneous antigen presentation. On day 14, as the tumor size increased over time, much greater CFSE dilution (48.3%) was present in the tumor-DLN. On day 21, the majority of T cells (61.9%) showed evidence of proliferation (Figure 3A).

At the same time, we analyzed the effector function of tumor-specific T cells on days 7, 14, and 21 using flow cytometry and IFN- γ ELISpot. On day 7, DLN cells from tumor-challenged mice showed a substantial number of IFN- γ -producing cells in response to irradiated B16 cells. However, the frequency of functional T cells diminished over time, such that by day 21 only background numbers of IFN- γ -spots were observed (Figure 3B). Consistent results were obtained in flow cytometry analysis of IFN- γ -releasing CD8⁺ T cells (Figure 3C). This result suggested that although T cell continued proliferate in tumor-DLN of lymphopenic mice, functional tumor antigen-specific CD8⁺ T cells wane over time.

In vivo T-cell hyporesponsiveness in later stage of T-cells homeostatic proliferation were attributed to the upregulation of PD-1 expression.

In order to understand the mechanism of T-cell hyporesponsiveness in later stage of T-cells homeostatic proliferation, we conducted time course experiments to determine the dynamic change of PD-1 expression on CD8⁺ T cells during recovery from lymphopenia condition. Barely detectable before transfer, PD-1 expression slightly increased at day 7, peaked at day 21, and returned to low levels by day 35 (Figure 3D). As the PD-1⁺ cell frequency declined from day 21 to 35, the total donor cell number moderately increased, indicating substantial loss in PD-1⁺ T cell number.

To examine whether cell death was involved in the process of homeostatic proliferation, CD8⁺ donor cells were stained with BCL-2 and Annexin-V on day 21 after transfer. BCL-2, an anti-apoptosis protein, can inhibit cell death induced by many cytotoxic factors. The downregulation of its expression indicate cell apoptosis. As shown in figure 3, the expression of BCL-2 significantly decreased while the percentage of apoptosis T cells increased on day 21 after transfer (Figure 3E & 3F). The correlation analysis showed that PD-1 expression on CD8⁺ T cells was significantly negatively related to the killing activity of cytotoxic T lymphocytes and the expression of BCL-2 on CD8⁺ T cells, but positively related to the percentage of apoptosis T cells (Figure 3G). These results suggested that T cells transferred to lymphopenic host highly expressed PD-1 and tend to apoptosis on day 21 of homeostatic proliferation.

Anti-PD-1 antibody treatment enhanced homeostatic proliferation–driven antitumor responses.

We next evaluate the therapeutic efficacy of anti-PD-1 antibody during reconstitution of the lymphopenic environment. Since PD-1 expression began to rise on day 7 after T cell infusion, we chose this time point to start anti-PD-1 antibody treatment. As shown in figure 5, anti-PD-1 antibody treatment significantly inhibited tumor growth and extended mouse survival time in immune reconstitution mice (Figure 4A-C). Moreover, anti-PD-1 antibody treatment significantly reduced the pulmonary metastasis in lymphopenic mice intravenously inoculated with B16 cells (Figure 4D).

Flow cytometry analysis of IFN- γ -releasing cells and IFN- γ ELISpot were performed to evaluate the antitumor immunity after anti-PD-1 antibody administration. The percentage of IFN- γ -releasing CD8⁺ T cells on Day21 was significantly increased in lymphopenic mice treated with anti-PD-1 antibody after T cell infusion (Figure 4F & 4G). Tregs and MDSCs, as the immunosuppressor cells, suppress antitumor immunity and weaken therapeutic efficacy of immunotherapy. To investigate the effects of anti-PD-1 antibody treatment on Tregs and MDSCs during recovery from lymphopenia, we assessed the percentage of MDSCs and Tregs in tumor-DLNs in lymphopenic mice treated with anti-PD-1 antibody after T cell infusion. Anti-PD-1 antibody treatment significantly reduced the recruitment of Tregs and MDSCs in immune-reconstituted mice (Figure 5A-C). These data revealed anti-PD-1 antibody treatment significantly enhanced homeostatic proliferation–driven antitumor responses.

Block of PD-1/PD-L1 axis extended the survival of homeostatic proliferation–driven CD8⁺ T cell and promoted it turn into memory T cells.

Previous studies have demonstrated that IL-7 or IL-15 plays an important role in the homeostatic expansion and survival of T cells and indicates the trend of tenting into memory T cells, so we analyzed whether the anti-PD-1 antibody treatment can increase the expression levels of IL-7R (CD127) and IL-15R (CD122) in CD8⁺ T cells. As expected, the expression levels of IL-7R and IL-15R were significantly increased after anti-PD-1 antibody administration (Figure 5E & 5F). It indicated the survival of T cell in the later stage of homeostatic proliferation.

T cells under homeostatic proliferation usually have two outcomes, apoptosis or turning into memory T cells, at the later stage of homeostatic proliferation. In order to explore whether anti-PD-1 antibody treatment can promote homeostatic proliferation–driven T cells to turn into memory T cells, the ratio of memory T cells were analyzed on day 21 after transfer. CD3⁺CD8⁺CD44^{high}CD62L^{high} cells, as central memory T cells, were significantly increased in lymphopenic mice combined treated with anti-PD-1 antibody and T cell infusion (Figure 5D).

Block of PD-1/PD-L1 axis promoted antigen recognition of HP cells during reconstitution of the lymphopenic environment.

The high ability of TAAs recognition is the important characteristic of HP T cells during the reconstitution of the lymphopenic environment. To evaluate whether block of PD-1/PD-L1 axis can enhance the antigen recognition ability of HP cells, functional status of DC cells were assessed using flow cytometry on day 14 after transfer. CD80 and CD86 were co-stimulatory molecule which highly express in activated DC cells, and I-A/IE was the MHC-II molecular which express in DC cells loaded with tumor antigen. As shown in figure 7, the expression of CD80, CD86 and I-A/IE in DC cells were significantly increased lymphopenic mice combined treated with anti-PD-1 antibody and T cell infusion when compared with anti-PD-1 antibody or T cell infusion alone (Figure 6A-C). These data suggested that block of PD-1/PD-L1 can enhance the function of DC cells and promote TAAs recognition during the period of recovery from lymphopenic condition.

A vaccine with TAAs give lymphopenic mice a tumor antigen for presentation and induce tumor-reactive memory T cells during homeostatic proliferation of T cells. To further assess the effects of anti-PD-1 treatment on TAAs recognition and memory response, we preimmunized lymphopenic mice with s.c. injection of irradiated B16 cells on the day of first anti-PD-1 antibody administration. As depicted in Figure 6D, tumor growth was slightly inhibited in preimmunized mice when living B16 cells was s.c. inoculated. Interestingly, anti-PD1 treatment notably enhanced antitumor efficacy and inhibited tumor growth by promoting TAAs recognition and the conversion of memory T cells (Figure 6D).

Crispr/Cas9-mediated knockout of PD-L1 in melanoma cells enhanced homeostatic proliferation–driven antitumor responses.

PD-L1 is a transmembrane protein, which is expressed on tumor cells, binds to PD-1 protein on T cells and inhibits T cell-mediated immune response. Treatment targeting the PD-1/PD-L1 pathway promote anti-tumor immunity and have shown promising results in some types of cancers. We next determined the kinetics of PD-L1 expression on tumor cells after which B16 cells were inoculated into lymphopenic C57/B6 mice. B16 cells have no expression or low expression of PD-L1 in vitro, but when they were inoculated into C57/B6 mice, the expression of PD-L1 rose smartly and peaked at day 7 due to the effects of immune microenvironment.(Figure 7A)

To further assess the role of PD-L1 on homeostatic proliferation–driven antitumor immunity, we next use Crispr/Cas9 system targeting PD-L1 gene for the generation of B16 cell lines with constitutive knockout of PD-L1 expression (Figure 7B & 7C). When PD-L1 knocking out B16 cells (B16-PD-L1) and B16 cells transfected with a control vector (B16-NC) were inoculated into immunocompetent C57BL/6 mice, knockout of PD-L1 significantly decreased tumor burden in irradiated mice after T cells infusion. (Figure 7D)

Crispr/Cas9-mediated knockout of PD-1 in T cells enhanced homeostatic proliferation–driven antitumor responses.

In immunocompetent hosts, adoptive transfer of PD-1 knockout T cells mediated by Crispr/Cas9 technology has been used in various cancers and got satisfactory treatment effectiveness [15, 16]. In lymphopenic mice, the lymphopenic condition may be able to create an environment to promote the PD-1 knockout T cells to recognize tumor antigen and undergo homeostatic proliferation, thus induced a more powerful antitumor immunity than adoptive transfer into immunocompetent hosts. To test this hypothesis, we designed and synthesized three crRNA/tracrRNA targeted murine PD-1 gene (Supplementary Table 1). The crRNA/tracrRNA was mixed with Cas9 protein to generate a Crispr/Cas9 ribonucleoprotein particle (Crispr/Cas9 RNPs). The Crispr/Cas9 RNPs were electroporated into nucleus to specifically knockout the PD-1 gene of T cells. The knockout efficiency was detected by T7E1 assays. The KO2 sequence showed maximum knockout efficiency and was selected for next experiment (Supplementary Figure 1).

Then the PD-1 knockout T cells were transferred into lymphopenic mice after 6Gy total body irradiation. As expected, tumor growth in lymphopenic mice intravenously infused PD-1 knockout T cells were significantly inhibited compared with transferring naïve T cells into lymphopenic mice or transferring PD-1 knockout T cells into immunocompetent mice.(Figure 7G) . These results indicated that the lymphopenic condition induced by total body irradiation may remarkably enhance the therapeutic effects of adoptive immunotherapy of PD-1 knockout T Cells.

Discussion

Total body irradiation (TBI) has strong immunomodulatory effects. At lethal dose, TBI is cytotoxic and lymphoablative, causing severe immune suppression and making mice die. At sub-lethal doses, TBI exhibits multifaceted immune-potentiating effects[17]. It has been shown that TBI can induce

immunogenic tumor cell death that enhances antigen presentation[18]. In addition, immunosuppressive cell, such as Tregs or MDSCs, and anergic immune cells were clear upon irradiation and tumor-induced immune tolerance was broken. More importantly, lymphopenic condition after total body radiation could create an environment to induce an enhanced antitumor response through the homeostatic proliferation of remaining T cells [19]. However, patients treated with radiotherapy or chemotherapy didn't show markedly enhanced antitumor immunity during the recovery period of myelosuppression. Either mechanisms exist to prevent LIP-induced antitumor response was as yet unknown.

In this study, we found that PD-1/PD-L1 play an important role on the reconstitution of immune tolerance against tumor after total body irradiation. We first observe that tumor growth was significantly inhibited when T cells were infused to irradiated mice, and CD8⁺ T cells were the main lymphocyte subsets responsible for the transfer of the antitumor immunity. The lymphopenic condition promoted exogenous or endogenous T cells undergo several rounds of proliferation. In this process, antitumor immunity was driven by the recognition of TAAs and the expansion of tumor reactive T cells.

Despite significant tumor suppression in irradiated mice during the early post-infusion period, this effect was not sustained in mice without anti-PD-1 treatment, because the tumor rapidly regained its growth. Borrello et al also found that antitumor immunity induced by homeostatic proliferation of tumor-specific T cells seem to rapidly decline in association with tumor growth[18-20]. The main reason for failure to completely eliminate the tumor might be the tolerance mechanism of T cells, such as exhaustion and anergy, and this might be influenced by the PD-1/PD-L1 axis. To avoid exhaustion of immune function, attempts at sustaining antitumor immune response through anti-PD-1 treatment should be made before the formation of immune tolerance, that is, long before full recovery from lymphopenic condition.

In this study, we found that despite the continuous proliferation of T cells in tumor-DLN of lymphopenic mice, the cytotoxicity of tumor reactive CD8⁺ T cells rapidly decline over time. To understand the mechanism of T-cell hyporesponsiveness in later stage of T-cells homeostatic proliferation, we conducted time course experiments to determine the dynamic change of PD-1 expression on CD8⁺ T cells during recovery from lymphopenia condition. We found that PD-1 expression on CD8⁺ T cells slightly increased on day 7, peaked on day 21, and declined to low levels on day 35. Moreover, the expression of PD-1 was closely related with the apoptosis and hyporesponsiveness of T cells in later stage of T-cells homeostatic proliferation.

Anti-PD-1 antibody has been widely used in multiple tumors and showed good therapeutic results. But a small percentage of patients fail to respond to anti-PD-1 antibody due to the influence of high tumor burden, tumor immunosuppressive microenvironment and other immunosuppressive signals. Exhausted effector T cells can be effectively activated by anti-PD-1 antibody, but they were taken into exhausted state quickly by other immunosuppressive signals in tumor microenvironment[21, 22]. In this study, we found that new microenvironment after immune reconstitution can enhance the therapeutic effects of anti-PD-1 antibody treatment. Combined treatment of immune reconstitution and anti-PD-1 antibody significantly inhibited tumor growth and extended mice survival time.

CRISPR/Cas9 technology is a recently developed gene editing platform, which has attracted great interest due to its simple application and high efficiency [23]. Adoptive transfer of PD-1 knockout T cells mediated by CRISPR/Cas9 technology has been used in various cancers and got satisfactory treatment effectiveness[16, 24-26]. However, the effectiveness is limited due to the low proliferation ability, antigen recognition ability and short lifetime of T cells in hosts. In this study, we found that the lymphopenic condition induced by total body irradiation may be able to create an environment to promote the PD-1 knockout T cells to recognize tumor antigen and undergo homeostatic proliferation, thus induced a more powerful antitumor immunity than adoptively transferring into immunocompetent hosts.

Conclusion

We found that PD-1/PD-L1 axis played an important role on reformation of immune tolerance after immunologic reconstitution. There is a synergistic action between anti-PD-1 antibody treatment and immune reconstitution on inhibiting tumor growth and enhancing antitumor immunity. Adoptively transferring PD-1 knockout T cells into lymphopenic mice has a better therapeutic effect than transferring into immunocompetent hosts on malignant melanoma. These findings has elucidated the mechanism of the wane of homeostatic proliferation–driven antitumor responses, and provided some guidance for developing novel combined treatment of anti-PD-1/PD-L1 therapy and large dose of radiotherapy or chemotherapy.

Abbreviations

BCL-2	B-cell lymphoma-2
Cas9	CRISPR-associated protein 9
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR-derived RNA
CTL	Cytotoxic lymphocyte
CTX	Cyclophosphamide
DLN	Draining lymph node
FACS	Fluorescence activated cell sorting
MACS	Magnetic-activated cell sorting
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
RNPs	Ribonucleoprotein particles
sgRNA	Small guide RNA
TAAAs	Tumor associated antigens
TBI	Total body irradiation
TCM	T central memory cells
tracrRNA	Trans-acting CRISPR RNA
Tregs	Regulatory T Cells
MDSCs	Myeloid-derived suppressor cells

Declarations

Ethical Approval and Consent to participate

Animal studies were carried out according to the institutional guidelines and approved by the Animal Care and Use Committee of Southern Medical University, China

Consent for publication

The article is approved by all authors for publication.

Availability of data and material

All data generated or analyzed during this study are included in the manuscript, figure, and supplementary files.

Competing interests (Mandatory)

All authors declare no financial and non-financial competing interests.

Funding

This work was supported by Science and Technology Program of Xiamen, China (No. 3502Z20194018) and National Natural Science Foundation of China (No. 81372449)

Authors' contributions (Mandatory)

ZY participated in the design of the study, performed the statistical analysis and drafted the manuscript. LY carried out the flow cytometric analysis. ZC carried out cell culture and CRISPR/Cas9 assay. LN participated in animal experiments. RL participated in the design of the study and revised the manuscript. SK conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements

We thank Weiyi Fang and Yuru Meng at Southern Medical University, Guangzhou, China.

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Figures

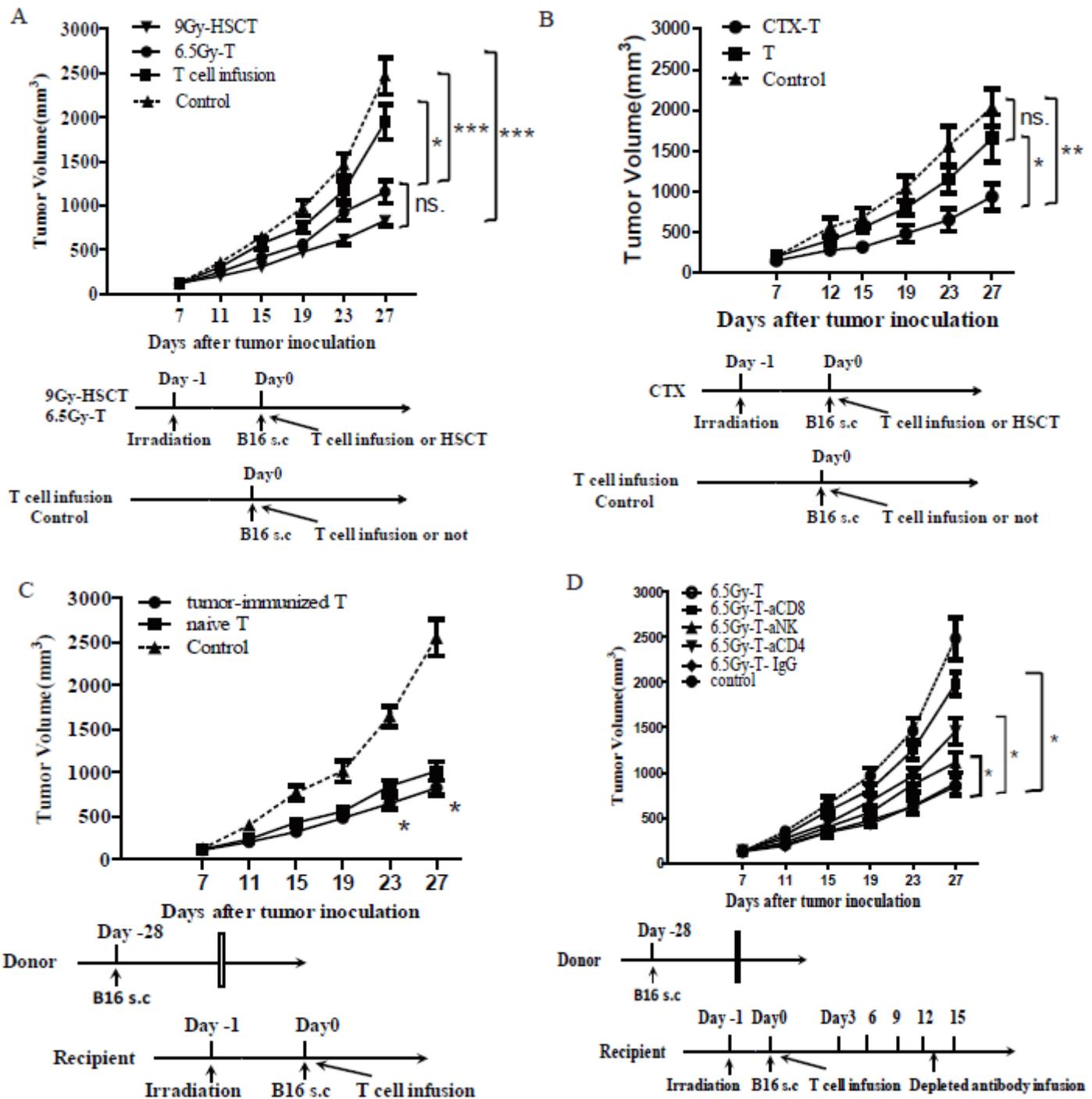


Figure 1

Growth suppression of malignant melanoma in lymphopenic hosts is depended on CD8+ T cells from donor mice. A. growth suppression of B16 s.c. tumor in lymphopenic mice. 9Gy-HSCT: The mice were irradiated with 9Gy (lethal) rays and received HSCT. 6.5Gy-T: The mice were irradiated with 6.5Gy (sublethal) rays and received T cells transfer. As a control, nonirradiated mice were untreated (control) or received T lymphocyte infusion (n=6). B. CTX-T: The mice were peritoneal injected with cyclophosphamide and received T cells transfer. C. The antitumor effect in lymphopenic mice received adoptive transfer of tumor-immunized splenocytes. The mice were sublethal irradiated and received T cell

infusion from tumor-bearing mice or naive mice (n=6). D. The antitumor effect of sublethal irradiation combined with T cell infusion with or without the depletion of CD8+ or CD4+ T cells, or NK cells. The T cells from donor mice were treated with anti-CD4, anti-CD8, and anti-asialo GM1 antibodies to deplete these cell populations before infusion (n=5). *, P<0.05. **, P<0.01. ***, P<0.001.

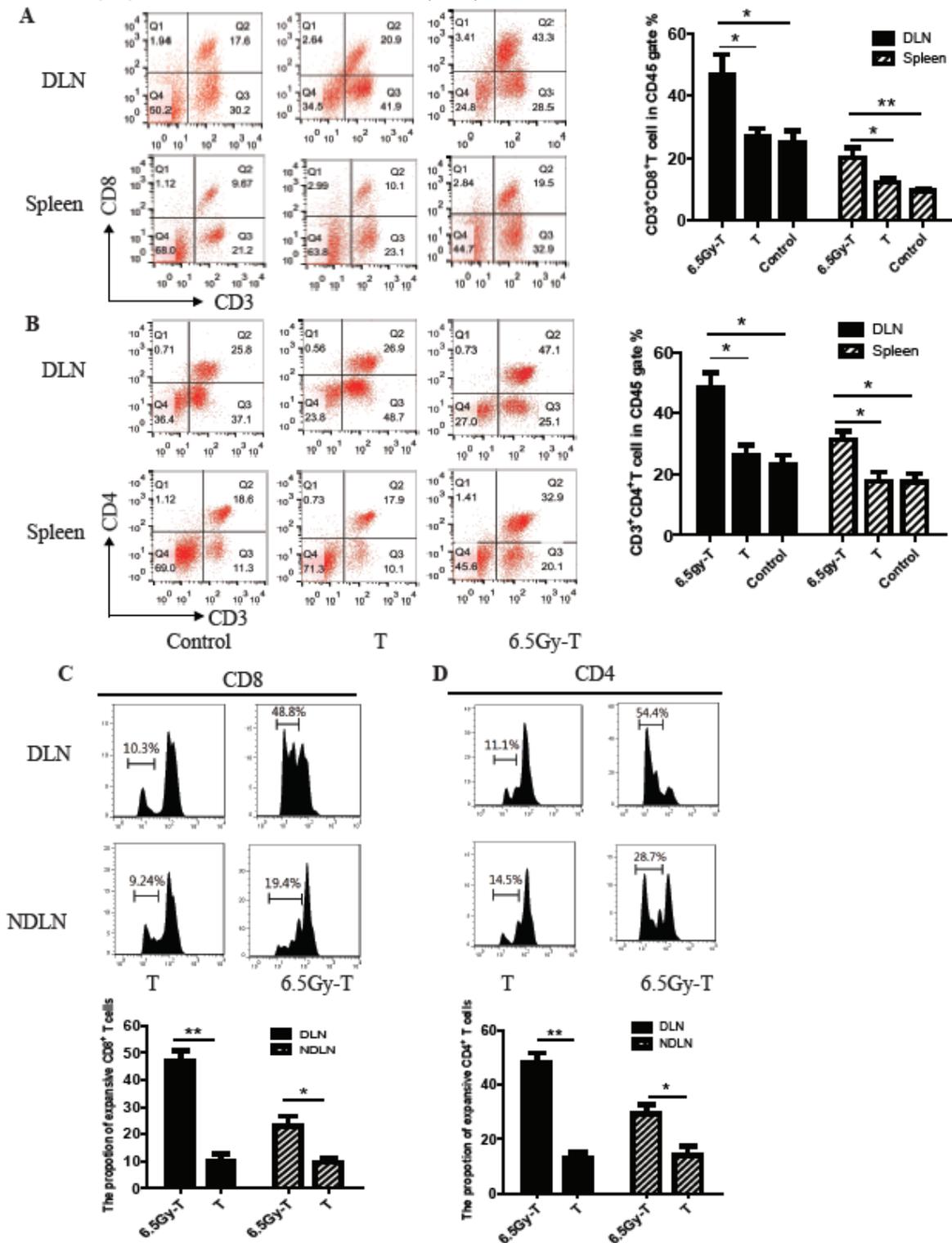


Figure 2

The homeostatic proliferation of T cells in lymphopenic mice. A-B. Flow cytometry analysis of CD3+ CD8+ T lymphocytes (A) and CD3+CD4+ T lymphocytes (B) in CD45+ gate in tumor-DLN and spleen(n=5). C-D. CFSE dilution of CD4+ and CD8+ T cells in tumor-DLN or tumor-NDLN. Splenic T cells labeled with CFSE were infused to lymphopenic mice after sublethal irradiation. CD8+ (C) and CD4+ (D) T cells were isolated to analyze the CFSE dilution by flow cytometry after 10 days. (n=5) *, P<0.05. **, P<0.01.

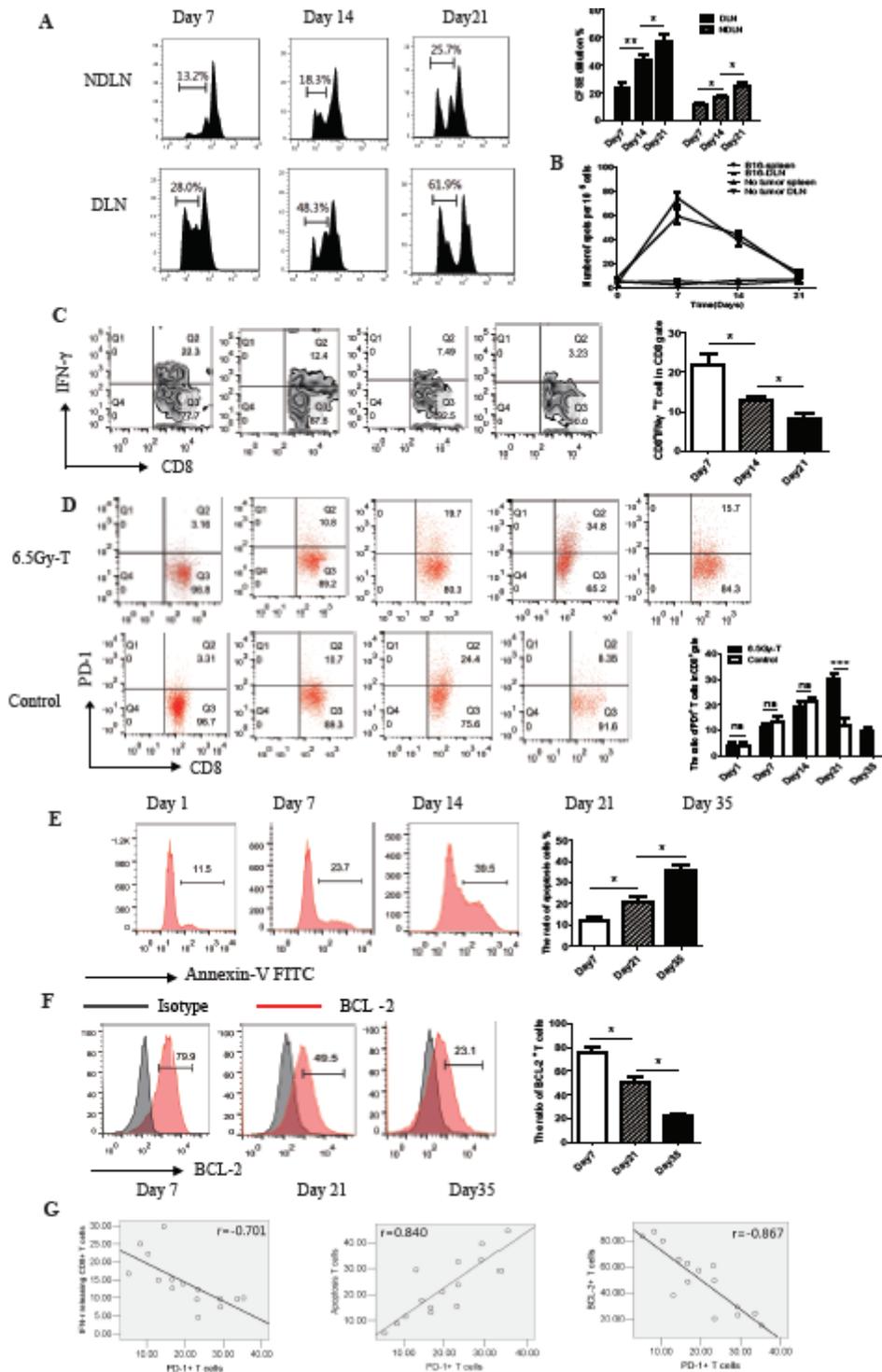


Figure 3

In vivo T-cell hyporesponsiveness in later stage of T-cells homeostatic proliferation were attributed to the upregulation of PD-1 expression. (A) CFSE dilution of CD8+ T cells in tumor-DLN and NDLN at the time point of Day7, 14 and 21. (B) Tumor-DLN were harvested from tumor-bearing mice at the indicated time points, and DLN cells were restimulated in vitro with the irradiated B16 cell and assayed by ELISpot for evidence of function as indicated by IFN- γ production.(C) Flow cytometry analysis of IFN- γ -releasing CTL in tumor-DLN on day7, 14, 21. (D) The kinetics of PD-1 expression on CD8+ T cells during reconstitution of the lymphopenic environment. E-F. The ratio of apoptosis cells (E) and BCL-2+ T cell (F) in CD8+ T cells at the time point of day 7, 21 and 35. (G) Pearson correlation analysis were performed between the percentage of PD-1+ T cell and the percentage of IFN- γ -releasing CD8+ T cells, apoptosis T cells and BCL-2+ T cells. *, P<0.05. **, P<0.01. ***, P<0.001.

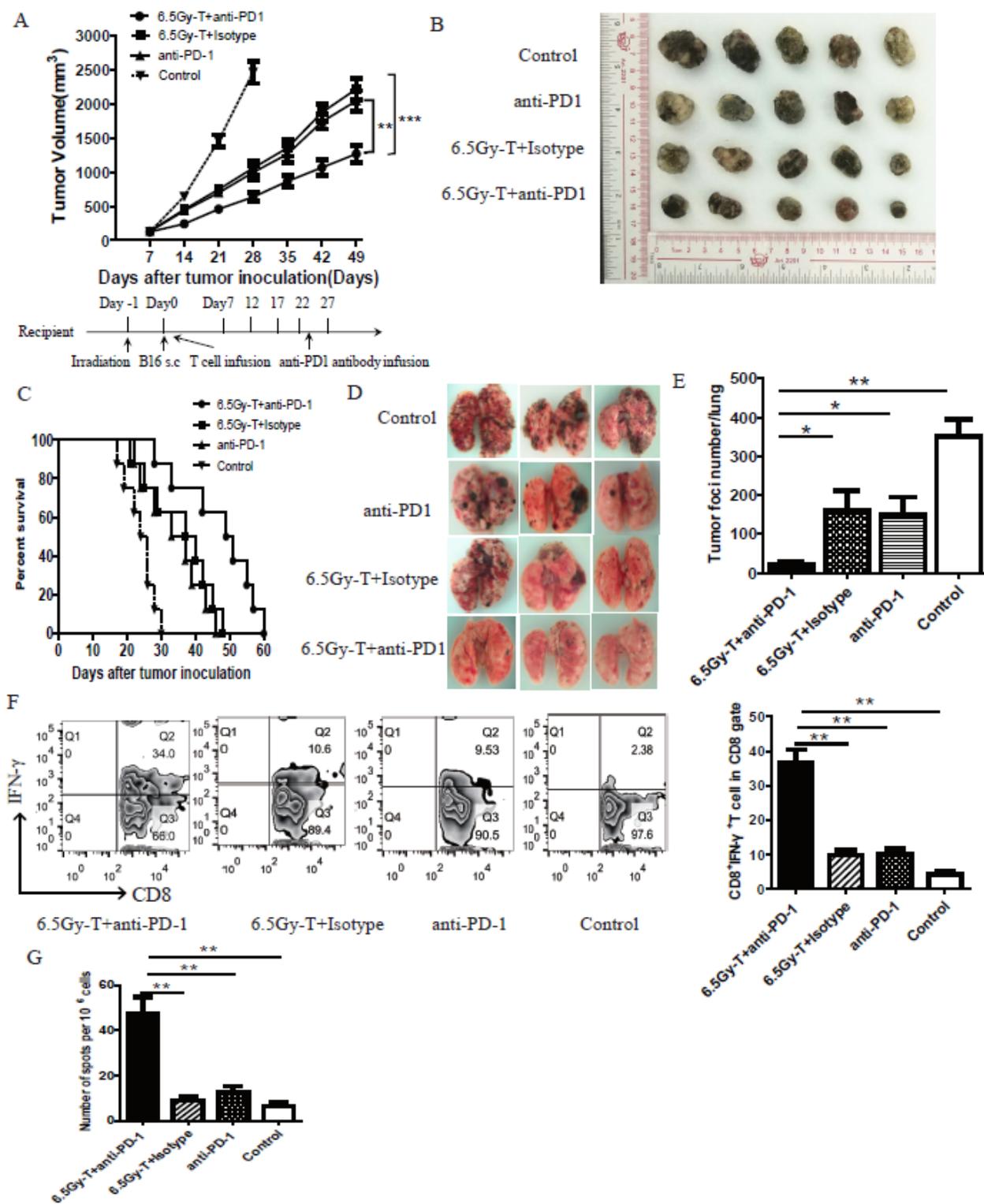


Figure 4

Anti-PD-1 antibody treatment enhanced homeostatic proliferation-driven antitumor responses. (A) Tumor growth curve in lymphopenic mice treated with anti-PD-1 antibody after T cell infusion. (B) The representative five subcutaneous tumor in each groups were shown. (C) Overall survival rates in lymphopenic mice treated with anti-PD-1 antibody. (D) The number of lung tumor foci in lymphopenic mice treated with anti-PD-1 antibody. The representative three lung tumor was shown. (E) IFN- γ ELISPOT

assay were performed in tumor-DLN cells in lymphopenic mice treated with anti-PD-1 antibody. (F) Flow cytometry analysis of IFN- γ -releasing CTL in tumor-DLN after anti-PD-1 antibody administration. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

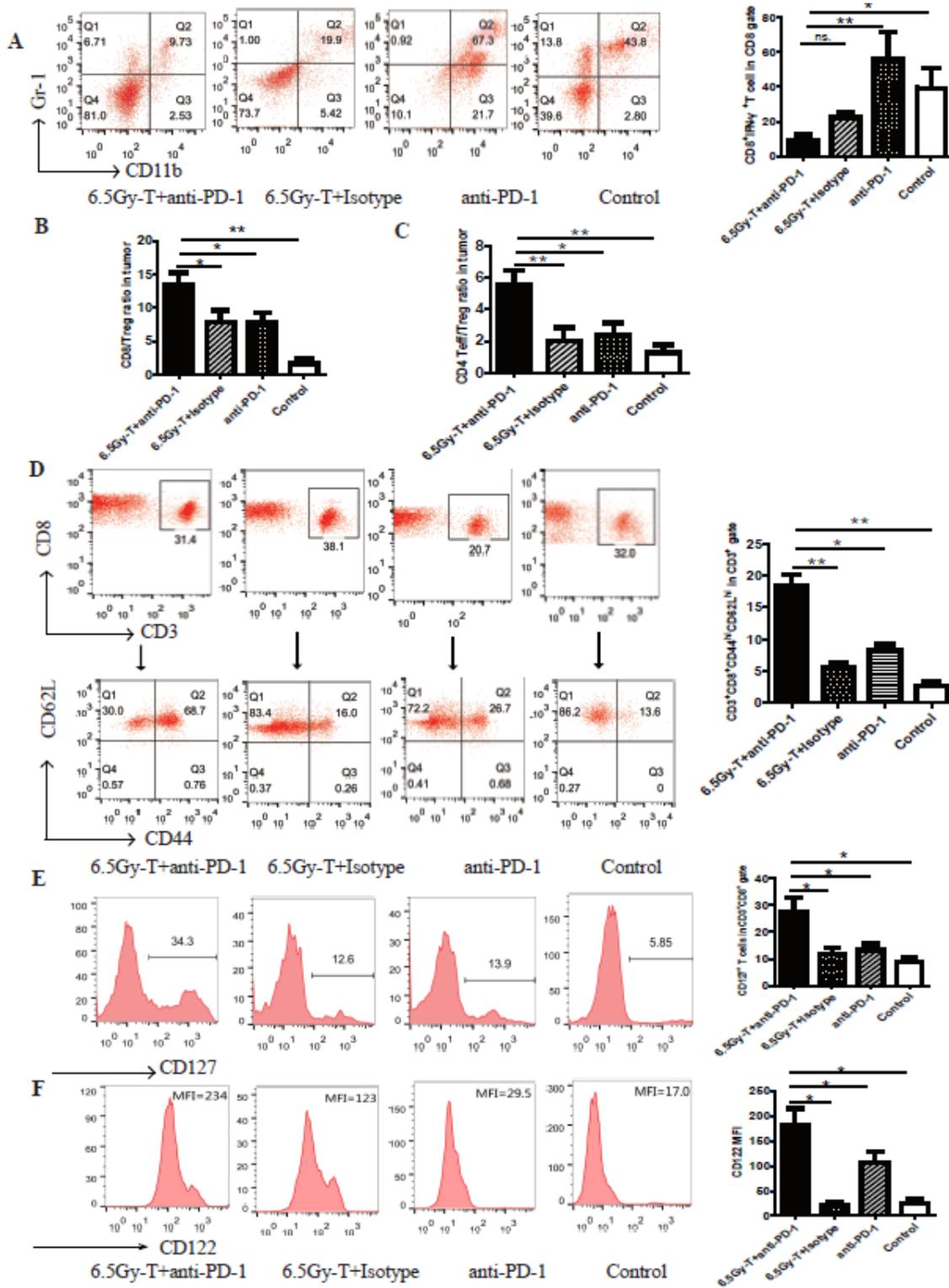


Figure 5

Block of PD-1/PD-L1 axis extended the survival of homeostatic proliferation-driven CD8+ T cell and promoted it turn into memory T cells. (A) Flow cytometry analysis of CD11b+Gr-1+ MDSCs on CD45+

gate in tumor-DLNs. (B) CD8+ T cells to Tregs ratios in tumor-DLNs. (C) CD4+ Effector cells to Tregs ratios in tumor-DLNs. (D) Flow analysis of CD3+CD8+CD44[high]CD62L[high] in CD3+ gate in tumor-DLN of lymphopenic mice combined treated with anti-PD-1 antibody and T cell infusion. (E) The percentage of CD127+ T cells in CD8+ gate in in tumor-DLN. (F) The mean fluorescence intensity (MFI) of CD122 in CD8+ gate in in tumor-DLN. *, P<0.05. **, P<0.01.

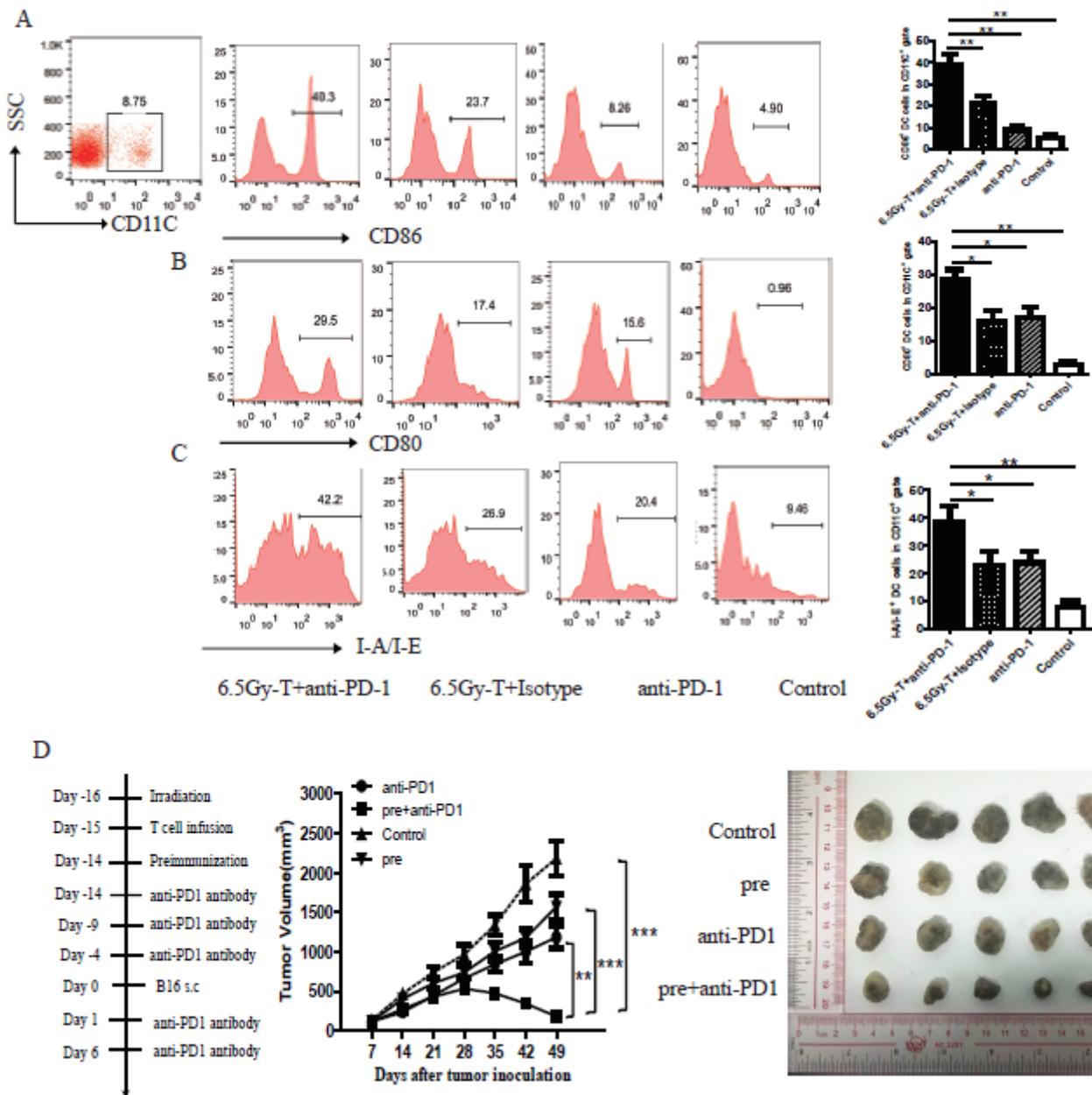


Figure 6

Block of PD-1/PD-L1 axis promoted antigen recognition of HP cells during reconstitution of the lymphopenic environment. (A-C). The percentage of CD86+(A), CD80+(B), I-A/I-E+(C) of DC cells in in tumor-DLN of lymphopenic mice combined treated with anti-PD-1 antibody and T cell infusion. D. Tumor growth curve in lymphopenic mice combined treated with irradiated B16 vaccine, anti-PD-1 antibody and T cell infusion. The representative five melanoma tumor was shown. *, P<0.05. **, P<0.01. ***, P<0.001.

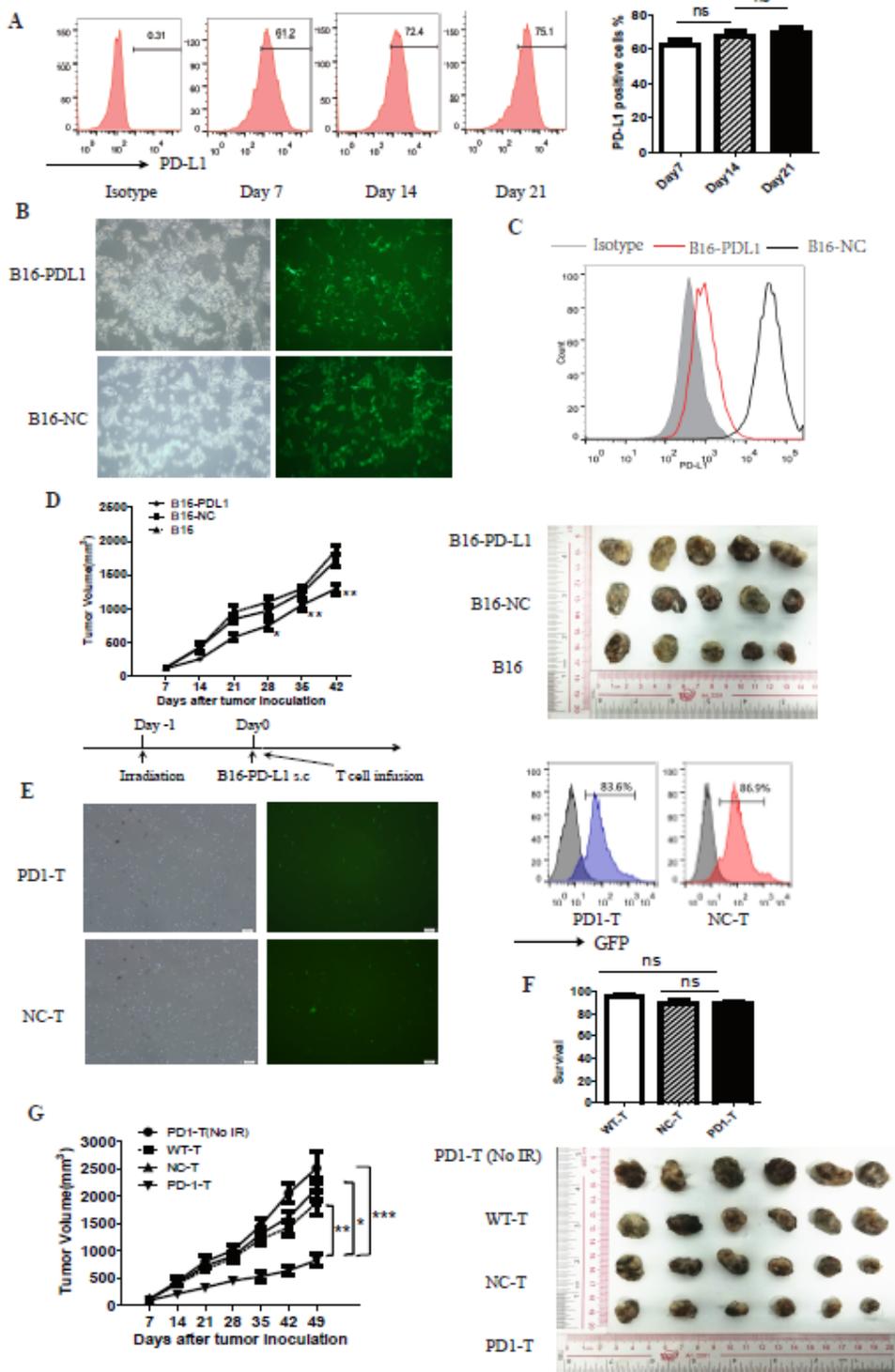


Figure 7

Crispr/Cas9-mediated knockout of PD-1 in T cells or PD-L1 in melanoma cells enhanced homeostatic proliferation-driven antitumor responses. (A) The kinetics of PD-L1 expression on CD45⁺ B16 cells during reconstitution of the lymphopenic environment. (B) The fluorescence photos of B16 cells transfected with PD-L1 k.o. lentivirus. (C) The expression of PD-L1 on B16 after B16-PDL1 and B16-NC cells were inoculated into irradiated mice. (D) Tumor growth curve of B16-PD-L1 and B16-NC cells in irradiated mice

after T cells infusion. The representative five lung tumor was shown. (E) The fluorescence photos of T cells after Crispr/Cas9 RNPs were electroporated into nucleus. Flow cytometry analysis of GFP expression of T cells after electroporation. (F) Survival of T cells was determined by trypan blue staining after electroporation. (G) Tumor growth curve in lymphopenic mice intravenously infused PD-1 knockout T cells. The representative six melanoma tumor was shown. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$. “ns”, no statistical significance.

Supplementary Files

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