Progranulin Induces Immune Escape in Breast Cancer Via Up-Regulating PD-L1 Expression on TAMS and Promoting CD8+T cell Exclusion

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Research

Keywords: Breast cancer, Progranulin (PGRN), TAMs, PD-L1, T cell

Posted Date: September 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-79474/v1

Version of Record: A version of this preprint was published on January 4th, 2021. See the published version at https://doi.org/10.1186/s13046-020-01786-6.

Abstract

Background: Progranulin (PGRN), as a multifunctional growth factor, is overexpressed in multiple tumors, but the role of PGRN on tumor immunity is still unclear. Here, we studied the effect of PGRN on breast cancer tumor immunity and its possible molecular mechanism.

Methods: The changes of macrophage phenotypes after PGRN treatment were detected by western blot, quantitative PCR and flow cytometry. Western blot was used to study the signal molecular mechanism of PGRN regulating this process. The number and localization of immune cells in WT and $PGRN^{-/-}$ breast cancer tissues were analyzed by immunohistochemical staining and immunofluorescence techniques. The activation and proliferation of $CD8^+$ T cells were measured by flow cytometry.

Results: After being treated with PGRN, the expressions of M2 markers and PD-L1 on macrophages increased. STAT3 signaling pathway inhibitor Stattic could significantly inhibit PD-L1 expression and M2 related markers induced by PGRN. In WT group, CD8 were co-localized with macrophages and PD-L1, but not tumor cells. The number of immune cells in $PGRN^{-/-}$ breast cancer tissue increased, and the infiltration into tumor parenchyma also increased. Moreover, in the co-culture system, WT peritoneal macrophages not only reduced the ratio of activated $CD8^+$T cells but also reduced the proportion of proliferating $CD8^+$T cells. The addition of PD-1 and PD-L1 neutralizing antibodies could effectively reverse this effect and restore the immune function of $CD8^+$T cells.

Conclusion: The results show that PGRN can promote M2 polarization and PD-L1 expression by activating the STAT3 signaling pathway. Furthermore, through PD-1/PD-L1 interaction, PGRN can promote the breast tumor immune escape. Our research may provide new ideas and targets for clinical breast cancer immunotherapy.

Background

In recent years, as an emerging treatment, breast cancer immunotherapy has attracted a lot of attention [1], especially systemic therapies mediated by programmed death ligand 1 (PD-L1) [2]. However, the immune escape generated during treatment raises a new challenge to its clinical efficacy [3].

PD-L1 can cause T cell dysfunction and failure, prevents cytotoxic T cells from effectively targeting tumor cells via binding to the programmed death receptor 1 (PD-1) on T cells, and thus promotes the occurrence and development of tumors [4, 5]. In recent years, research on PD-L1 has mainly focused on tumor cells [6]. However, interestingly, there have been reports that tumor-associated macrophages (TAMs) are the main source of PD-L1 expression in the tumor microenvironment (TME). The expression of PD-L1 on TAMs is longer and more stable than those on tumor cells [7].

Tumor-associated macrophages, which are one of the main components of tumor infiltrating immune cells, play a crucial part in tumor invasion and metastasis [8]. According to different activation signals, they are mainly classified as M1 and M2 phenotype. Usually, TAMs are dominated by M2 macrophages

that promotes tumor progression in the tumor microenvironment [9]. Prostaglandin E2 (PGE2), IL-13, statins, etc. can regulate the polarization of macrophages to M2-like TAMs [10]. Recent studies have reported that progranulin (PGRN) also has something to do with the macrophage polarization [11, 12]. However, there is little report about how PGRN affects the polarization and function of macrophages in breast cancer.

Progranulin (PGRN) is composed of 593 amino acids [13], and participates in various pathophysiological processes including neurodegeneration, tissue damage repair, embryonic development and so on [14]. In the past, people paid more attention to the role of PGRN in inflammatory diseases [15]. But recently, researchers have shifted their attention to the relationship between PGRN and tumors [16]. Studies have reported that PGRN is relevant to the poor prognosis of patients with tumor [17]. Unfortunately, so far, the effect of PGRN on the development of breast cancer and the specific molecular mechanism have not been fully determined. In our study, we found that PGRN can not only promote macrophages to the M2-like TAMs, up-regulate the PD-L1 expression on TAMs, but also inhibit the $CD8^+$ T cell immune function.

In tumor immunity, $CD8^+$ T cells are the key cells against tumors [18]. They accumulate in tumor tissues, make physical contact with malignant tumor cells, and then kill tumors targetedly through activation signals from other cells [19]. Current studies have shown that a key factor limiting the success of cancer immunotherapy is the occurrence of "immune exclusion" in the tumor microenvironment, that is, the lack of T cells in the tumor region, where $CD8^+$ T cells are excluded from the vicinity of cancer cells, and then the $CD8^+$ T cell immune function is suppressed [20, 21]. Therefore, immune escape occurs, after tumor cells successfully evade immune attack [22].

This study is the first time to clarify the effect of PGRN on PD-L1 expression in TAMs. We discovered a novel mechanism for PGRN to promote breast cancer immune escape. We aimed to reveal the crucial function of PGRN in inducing immunosuppression in breast cancer, hoping that this study could provide new ideas for enhancing the clinical immune efficacy of breast cancer.

Methods

Cell lines and reagents

Raw264.7 cells were cultured in complete DMEM (Gibco) medium. PY8119 cells were cultured in DMEM:F12(1:1) (Gibco) supplemented with 10% FBS (Gibco). Macrophages were polarized in M1 macrophages with 100 ng/mL LPS (R&D Systems, USA) and 20 ng/mL IFN-γ (R&D Systems, USA); and macrophages were treated with 20 ng/mL IL-4 (R&D Systems, USA) to generate M2 macrophages.

Small interfering RNA (siRNA) (Invitrogen) for PGRN was transfected with R4000 (Engreen, China). Recombinant murine PGRN was purchased from RD (R&D Systems, USA). Stattic, LY294002 and U0126 were purchased from MCE (Shanghai, China).

Peritoneal macrophages isolation

Each mouse was injected intraperitoneally with 2 ml of 3% thioglycollate (Difco) on day 1. Mice were sacrificed 4 days after injection. Peritoneal macrophages were harvested from peritoneum, after being injected with 7ml cold PBS into peritoneal cavity. The peritoneal cells were centrifuged at 1500 RPM for 10 min then seeded into cell culture dishes. The suspension cells were discarded by washing with PBS after 2h. And the adherent cells were considered as peritoneal macrophages.

In vitro T cell activation assay and co-culture

Spleens were ground with syringes, washed with PBS, and then passed through 70 μm cell strainers to gain single-cell suspensions. Red blood cells were lysed by using Red Blood Cell Lysing Buffer (Biosharp, China). Splenocytes were further separated with mouse spleen lymphocyte separation solution (tbdscience, China) to obtain spleen lymphocytes. Obtained spleen lymphocytes were cultured in complete RPMI 1640 medium. For T-cell activation assays, anti-CD3e (5μg/ml; eBioscience) was pre-coated in 96 well plates overnight at 4°C. Subsequently anti-CD28 (1μg/ml; BD) was added to the plates.

For co-culture assay, peritoneal macrophages at indicated ratios were added to the medium after T cell activation. Moreover, cells were cocultured with or without neutralizing monoclonal antibodies against PD-L1, PD-1 or IgG isotype control (BioLegend). After 4 days, cells were detected by flow cytometry.

Immunohistochemistry

Sections from tumors were cut into 4 μm in thickness and deparaffinized in xylene for 10 min. The slides were immersed in 3% H_2O_2 for 20 min to block the endogenous peroxidase and were blocked in goat serum blocking solution for 30 min. After being incubated at 4 °C overnight with primary antibodies, the slides were incubated with secondary HRP-conjugated antibodies (Thermo Fisher Scientific) for 30 min at RT. The primary antibodies were as follows: F4/80 (Cell Signaling Technology), iNOS (Abcam), CD206 (proteintech), CD4 (Cell Signaling Technology), CD8 (Cell Signaling Technology), Granzyme B (NOVUS), and PD-1 (Abcam). IHC stainings were examined with microscopy.

Immunofluorescence

Slides from tumors were deparaffinized in xylene and dehydrated in graded ethanol solutions. The sections were blocked in goat serum blocking solution for 1h at room temperature. The slides were incubated overnight at 4 °C with the following antibodies for multicolor immunofluorescence staining: F4/80 (Cell Signaling Technology), PD-L1 (Abcam), CD206 (proteintech), Arg1 (Abcam), iNOS (Abcam), CD8 (Cell Signaling Technology), CD4 (Cell Signaling Technology), CK19 (Abcam), and PD-1 (Abcam). The next day, the slides were washed in PBS and stained with the secondary antibody for 1 h at room temperature. Multicolor immunofluorescence staining was detected with fluorescence microscope.

Western blot

The cells were lysed in RIPA lysis buffer (Beyotime, China) supplemented with protease inhibitors PMSF (Beyotime, China). Protein concentration were determined by using BCA protein assay kit (Beyotime,

China). The primary antibodies included iNOS (Abcam), Arg1 (Abcam), PGRN (Abcam), PD-L1 (Abcam), STAT3 (Cell Signaling Technology), pSTAT3 (Cell Signaling Technology), AKT (Cell Signaling Technology), pAKT (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), pERK1/2 (Cell Signaling Technology), and β-actin (proteintech).

RNA extraction and real-time quantitative PCR

Total RNA was extracted with TRIzol reagent (TaKaRa, Japan), and cDNA was reverse transcribed subsequently with PrimeScript RT reagent kit (TaKaRa, Japan). qRT-PCR was then performed using a SYBR Premix Ex Taq II (TaKaRa, Japan) according to the manufacturer's instructions. The sequences of primers were presented in Table 1.

Flow cytometry

The single-cell suspensions were first blocked with anti-CD16/32 (101302, BioLegend) for 10 min at 4 °C. Then the following antibodies were used: PE/Cy7-PD-L1, APC-CD206, PE-CD86, Brilliant Violet 421^{TM}-CD86, APC-CD8a, PE-IFN-γ, PE-Ki-67, and PE-PD-1 from Biolegend.

Orthotopic breast tumor model

Wild-type (WT) C57BL/6 mice aged 6 to 8 weeks were obtained from Chongqing Medical University. PGRN knock out (KO) mice were a generous gift from Dr. Yibing Yin. A total of 5×10^6 PY8119 cells suspended in 100 μl of PBS were injected into the fourth right mammary fat pad of the mice. Tumor growth was evaluated by measuring tumor volume ($TV=0.5\times length\times width^2$) every 3 days until they were sacrificed 3 weeks after treatment. Survival was determined by tumor size $\geq$1000 mm^3 or animal death. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University.

Statistical analysis

Unpaired student t-test was used for mean difference comparison between two groups. One-way ANOVA followed by multiple comparison was used for multiple groups. All data were presented as mean±SEM or SD. $P < 0.05$ was considered as significant. All the experiments were performed independently for three times.

Results

PGRN promotes the polarization of macrophages towards M2 phenotype

In order to clarify how PGRN affects the growth and metastasis in breast cancer, we first injected breast cancer cells PY8119 into the fat pad of mice in situ to construct a breast cancer model. Compared with

the WT group of mice, the breast tumors of PGRN$^{-/-}$ mice grew slower and had better survival (Fig. 1A, B). For the past few years, the tumor microenvironment (TME) has attracted much attention because it has been found to be closely related to the tumor progression [23, 24]. TME is composed of tumor cells and recruited stromal cells. Among them, as important immune cells, TAMs have important functions in TME [8, 25]. Therefore, we used immunohistochemical staining to test the expression of M1 and M2 markers in breast cancer tissue sections. In contrast, more F4/80 macrophages were infiltrated in the WT group. It is worth noting that the WT group had a low expression of classic M1 marker inducible nitric oxide synthase (iNOS), but a high expression of M2 marker mannose receptor 1 (CD206) (Fig. 1C). It is known that LPS can induce macrophages to be polarized to M1 phenotype, and IL-4 can induce them to M2 [26, 27]. In order to compare the polarization effect of PGRN with the classic inducer LPS and IL-4 on macrophages, we used western blotting and flow cytometry to detect M1 (CD86, iNOS) and M2 (Arg1, CD206) markers expression in macrophages treated with exogenous PGRN recombinant protein. We found that PGRN reduced iNOS and CD86 expression, and increased arginase 1 (Arg1) and CD206 expression (Fig. 1D; Supplementary Fig. 1A-B). Interestingly, PGRN can also reduce the induction of LPS on M1, and the combination of IL-4 and PGRN can significantly enhance the polarization of PGRN on M2 (Fig. 1D). Furthermore, PCR analysis indicated when the PGRN gene expression of RAW264.7 was disturbed, the expression of representative M1 gene *(IL-12)* and *(TNFα)* increased, while the expression of M2 gene *(Arg1)* and interleukin-10 *(IL-10)* decreased (Fig. 1E).

In order to further examine whether endogenous PGRN affects the polarization of macrophages, we treated WT and PGRN$^{-/-}$ peritoneal macrophages with LPS and IL-4 respectively. Interestingly, we found that WT peritoneal macrophages are more sensitive to IL-4 stimulation but not to LPS (Fig. 1F, G). This is consistent with the results of the RAW264.7 cell line, indicating that PGRN can promote the M2 macrophages polarization.

Pgrn Up-regulates Pd-l1 Expression On Tams

To ascertain how PGRN affects PD-L1 expression in M2, we first treated M2 with PGRN recombinant protein. Flow cytometry and PCR results showed that PGRN upregulated PD-L1 of M2 in a concentration-dependent and time-dependent manner (Fig. 2A, B). This was also verified by western blot at the same time (Supplementary Fig. 2A-B). It is noteworthy that PGRN can significantly up-regulate CD206^{+}PD-L1^{+} (Fig. 2E), which further suggests that PGRN does up-regulate PD-L1 of M2. Next, we measured the macrophage markers expression and their respective co-localization with PD-L1 through multicolor immunofluorescence staining. Compared with the PGRN$^{-/-}$ group, we found F4/80, CD206, and Arg1 expression in the WT group was significantly increased, but the expression of iNOS was lower (Fig. 2F), which is consistent with our IHC results (Fig. 1C). In addition, it is interesting that PD-L1 and F4/80, CD206 and Arg1 in the WT group were significantly co-localized, while the co-localization of iNOS and PD-L1 in the PGRN$^{-/-}$ group were more significant. (Fig. 2F). Then when we treated WT and PGRN$^{-/-}$ peritoneal macrophages with IL-4, we also found that the PD-L1 expression on WT peritoneal

macrophages was higher than that on PGRN$^{-/-}$, no matter at the protein or the mRNA transcription level. (Fig. 2C, D). The above results indicate that PGRN can up-regulate the PD-L1 expression on TAMs.

PGRN/STAT3 signaling pathway regulates the polarization of TAMs and up-regulates the PD-L1 expression

Next, to explore the signal pathway through which PGRN affects the polarization of TAMs and the PD-L1 expression, we tested the changes of STAT3/pSTAT3, AKT/pAKT and ERK1/2/pERK1/2 signal pathways after TAMs were treated with PGRN. As expected, WB results showed that PGRN could increase phosphorylation of STAT3, AKT and ERK1/2, but had no significant impact on total STAT3, total AKT and total ERK1/2 (Fig. 3A). After exposure to PGRN, phosphorylation of STAT3 could be detected at the 15th minute and increased thereafter. In addition, obvious phosphorylation of AKT and ERK1/2 could be observed after being stimulated with PGRN for 30 minutes (Fig. 3B). Next, in order to figure out the key role of STAT3, AKT, and ERK1/2 in PGRN-mediated M2 polarization and PD-L1 expression, we used signaling pathway inhibitors Stattic, LY294002, and U0126 to treat TAMs exposed to PGRN, respectively. Unexpectedly, only Stattic, an inhibitor of STAT3, could significantly inhibit the PD-L1 expression in TAMs induced by PGRN, and as the dose of Stattic increased, the inhibition of PD-L1 became more obvious (Fig. 3C, D). However, whether LY294002 (AKT inhibitor) or U0126 (ERK1/2 inhibitor) couldn't distinctly prevent the upregulation of PD-L1 (Fig. 3E, F). And interestingly, after treatment with signal pathway inhibitors, the expression changes of the M2 marker Arg1 were consistent with the PD-L1 response (Fig. 3C-F). These results indicate that PGRN can induce TAMs polarization and PD-L1 expression via activating STAT3.

PGRN promotes CD8 $^{+}$ T cell exclusion and inhibits tumor immunity

In order to further examine how PGRN affects tumor immune cells, by using immunohistochemical staining, we discovered the infiltration of CD4^{+} cells, CD8^{+} cells, and granzyme B^{+} cells in breast cancer tumor tissues in PGRN$^{-/-}$ group was more than those in WT group, especially CD8^{+} cells and Granzyme B^{+} cells. It is worth noting that the CD8^{+} cells in PGRN$^{-/-}$ group shifted from the tumor stroma to the tumor parenchyma (Fig. 4A). Multicolor immunofluorescence staining analyzed the co-localization of CD8^{+} cells with tumor cells, macrophages, and PD-L1^{+} cells. Interestingly, in WT group, CD8^{+} cells were scattered in the tumor stroma and there was a more obvious co-localization with F4/80^{+} cells, CD206^{+} cells, and PD-L1^{+} cells. Notably, in PGRN $^{-/-}$ breast cancer tissue sections, the co-localization of CD8^{+} cells and CK19^{+} tumor cells was significantly increased (Fig. 4B). The results above indicate that in breast cancer, PGRN can cause CD8^{+} T cell exclusion and affect the spatial distribution of CD8^{+} T cells, which may be related to TAMs or PD-L1 in TME.

Next, to explore whether PGRN affects T cell immune function, we co-cultured the splenic lymphocytes of C57 WT mice with WT or PGRN$^{-/-}$ peritoneal macrophages. It was worth noting that wild type peritoneal macrophages could not only inhibit the amount of IFN-γ in CD8^{+} T cells but also inhibit the expression of

proliferation antigen Ki-67 by $CD8^+$ T cells (Fig. 4C, D). These results indicate that PGRN can inhibit tumor immunity in breast cancer via facilitating the exclusion of $CD8^+$ T cells.

PD-1/PD-L1 axis mediates the immunosuppressive function of PGRN in breast cancer

In the tumor microenvironment, programmed death receptor 1 (PD-1) binds to its ligand PD-L1 to regulate T cell activation and inhibit T cell-mediated immune responses [28, 29]. To explore the molecular mechanism through which PGRN restrains the immune function of $CD8^+$ T cells in breast cancer, firstly, we discovered PD-1 was highly expressed in WT breast cancer tissues (Fig. 5A, B), The immunofluorescence results proved that PD-1 was co-localized with PD-L1 significantly. Notably, the infiltration of immune cells in WT group was prominently less than that in the $PGRN^{-/-}$ group, especially $CD8^+$ cells. And whether it is $CD8^+$ cells or $CD4^+$ cells, co-localization with PD-1 could be detected (Fig. 5B). When we co-cultured WT and $PGRN^{-/-}$ peritoneal macrophages with splenic lymphocytes of WT mice, the WT group could significantly increase the ratio of $CD8^+PD\text{-}1^+$ cells (Fig. 5C). Interestingly, when we added PD-1 neutralizing antibody or PD-L1 neutralizing antibody to the co-culture system, the immunosuppressive function of WT peritoneal macrophages on $CD8^+$ cells was distinctly reversed, especially the combination of PD-1 and PD-L1 blockade (Fig. 4C-D and Fig. 5D, E). The results above indicate that PGRN exerts an immunosuppressive function through PD-1/PD-L1 interaction.

Discussion

Macrophages contribute more than 50% of the tumor-infiltrating cells [30]. Infiltration of macrophages is closely correlated with poor prognosis of breast cancer [10, 31]. TAM suppresses the immune response through multiple mechanisms. For example, the aerobic glycolysis of tumor cells increases the production of lactic acid and induces the M2 polarization of TAM to antagonize the local immune response [32]. Macrophages can regulate their phagocytosis and antigen presentation function through PD-1/PD-L1 immune checkpoints, thereby promoting tumor cells to evade phagocytosis and clearance [33].

In our study, it's the first time to determine that PGRN can not only promote the polarization of macrophages to M2 phenotype, but also up-regulate the PD-L1 expression of TAMs. PGRN interferes with the interaction between TNFα and TNFR by competing with tumor necrosis factor α (TNFα) to bind to tumor necrosis factor receptor (TNFR) [34, 35]. And PGRN can activate intracellular protein kinase B (PKB/AKT) [36] and extracellular signal-regulated kinase (ERK) to promote cell proliferation, migration and malignant transformation [37]. As is known to all, there are various signal pathways concerning the post-transcriptional regulation of PD-L1. Among these signal pathways, the two most important ones are JAK/STAT/PI3K/AKT/MEK/ERK and SHP2/RAS/RAF/MEK/ERK pathways [38–40]. When we treated TAMs with PGRN, we found that their p-STAT3, p-AKT and p-ERK1/2 protein levels were increased. To this end, we used inhibitors Stattic, LY294002 and U0126 to block JAK/STAT3, PI3K/AKT and ERK1/2 pathways, respectively. The striking fact is that only after treatment with Stattic, the PD-L1 induction effect of PGRN on TAMs was significantly inhibited, and after the activities of p-AKT and p-ERK1/2 were

inhibited, the PD-L1 expression of TAMs did not change significantly. In addition, interestingly, we found that the polarization of M2 was also consistent with the changes in PD-L1. This indicates that in TAMs, PGRN up-regulates PD-L1 and promotes the polarization of M2 by activating the JAK/STAT3 signaling pathway. Our work in future will further explore whether PGRN regulates this process through other key signal transduction pathways.

Stromal cells in TME, such as cancer-associated fibroblasts (CAF), myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAMs) allow cancer cells to gain immune privilege through excluding T cells from nearby tumor cells [20]. There are research reports that MDSC prevents T cell recruitment and infiltration through the nitrifying chemokine CCL2, leading to T cell distribution in the matrix [41]. Indole 2,3-dioxygenase (IDO) produced by tumor cells and MDSC can not merely induce the transformation of Treg cells, but also decompose tryptophan to produce kynurenine acid, thereby restraining T cell proliferation [42]. In squamous cell carcinoma, B cells inhibit T cell recruitment via reprogramming macrophages. After B cell depletion, macrophages show an M1 inflammatory phenotype, and $CD8^+$T cell infiltration significantly increases [43]. In our study, we observed that in the breast cancer tissues of the WT group, $CD8^+$T cells were mainly distributed in the tumor stroma, and there was little co-localization with the tumor cell marker CK19. Immunofluorescence results showed that $CD8^+$T cells in the WT group were co-localized with F4/80, CD206, and PD-L1. When splenic lymphocytes were co-cultured with WT and $PGRN^{-/-}$ peritoneal macrophages, WT peritoneal macrophages could prominently inhibit the $CD8^+$T cell proliferation and killing activity, indicating that PGRN could inhibit breast cancer tumor immunity.

PD-L1 and PD-L2 are the two ligands of PD-1, both of which belong to the B7 family. Among them, PD-L1 is widely expressed in various hematopoietic and non-hematopoietic cells, while PD-L2 is usually lowly expressed and its expression is limited to activated dendritic cells, macrophages, bone marrow-derived mast cells and peritoneal B1 cells surface, so it is currently believed that the main ligand of PD-1 is PD-L1 [44, 45].

After PD-1 expressed on activated T lymphocytes binds to PD-L1, the immunoreceptor tyrosine-based inhibitory motif in the intracellular domain of PD-1 is phosphorylated and tyrosine phosphatase is recruited to enrich the intracellular domain. In the intracellular region, accumulated phosphatase dephosphorylates key proteins in the T cell antigen receptor (TCR) signaling pathway and inhibits its downstream signaling pathways such as PI3K/AKT/mTOR, and RAS/MEK/ERK [46, 47]. Ultimately, T cell proliferation, differentiation and cytokine production were hindered [28]. Currently, the PD-1/PD-L1 interaction leading to tumor immune escape is a research hotspot and a difficult point [48], which has not yet been fully explained. In this study, when we blocked PD-1 and PD-L1, the immunosuppressive functions of PGRN was distinctly rescued, especially the coadministration of PD-1 and PD-L1 antibody, the ratio of IFN-γ^+CD8^+T cells and Ki-67^+CD8^+T cells increased significantly. Taking together, our research shows that in breast cancer, PGRN may exert its immunosuppressive function via the PD-1/PD-L1 axis.

Next, further researches are needed to explore whether PGRN directly or indirectly affects the PD-1/PD-L1 interaction through other key molecules.

In conclusion, we have discovered a new role of PGRN to promote breast cancer progression, that is inducing M2 polarization and up-regulating PD-L1 through PGRN/STAT3, and then promoting $CD8^+$T cell exclusion and inhibiting tumor immunity through PD-1/PD-L1 interaction. This implies that PGRN is expected to become a new therapeutic target for breast cancer immunotherapy, and it may improve the efficacy of PD-1/PD-L1 antibody clinical immunotherapy.

Abbreviations

KO
Knock out
PGRN
Progranulin
PD-L1
Programmed death ligand 1
PD-1
Programmed death receptor 1
qPCR
quantitative PCR
TAMs
Tumor-associated macrophages
TME
Tumor microenvironment
WT
Wild-type

Declarations

Acknowledgments

We would like to thank Dr. Shifang Dong from Pediatric Research Institute of Children's Hospital of Chongqing Medical University for flow cytometry guidance. We also thank Zuochen Du (Chongqing Medical University) for advice and technical support.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81772844), and by a grant from the Graduate Students Science Innovation Project of Chongqing (No. CYS19206).

Availability of data and materials

The datasets analyzed in this study are available from the corresponding author on reasonable request. The datasets supporting the conclusions of this article are included within the article and its additional file.

Authors' contributions

WLF and TMC conceived and designed the study. WLF performed the experiments and drafted the manuscript. DZ, HSQ, FFJ, QZ, YGW, CSC contributed to manuscript editing. WLF, TZ, HS, MLY analyzed data and prepared the figures. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All authors provide their consent for publication of the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Chongqing Medical University.

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Figures

Fig1

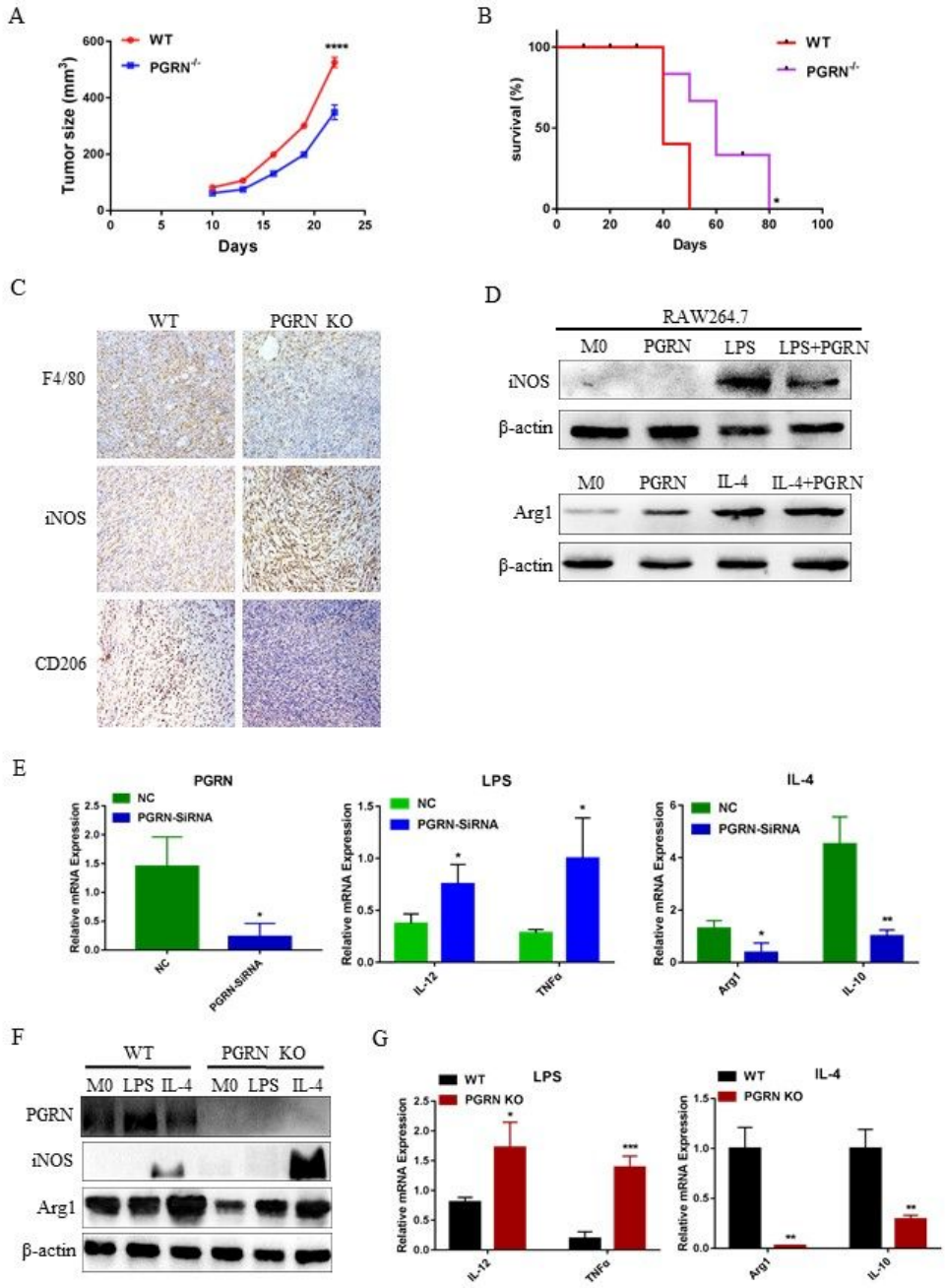

Figure 1

PGRN promotes M2 polarization of macrophages (A-B). Breast cancer PY8119 cells were injected in situ into the fat pads of C57 wild-type mice and PGRN knock out mice (n=5 per group). A. Tumor volume curve. B. Survival curve of mice. C. F4/80, iNOS and CD206 expression were detected by IHC in breast cancer tissue sections of WT and PGRN KO mice respectively. (D-E). RAW264.7 macrophage cell line was treated with PGRN recombinant protein and LPS or IL-4. D. iNOS and Arg1 expression were examined by

western blot. E. M1 markers (IL-12, TNF-α) and M2 markers (Arg1, IL-10) were tested by PCR. (F-G) WT and PGRN KO mouse peritoneal macrophages were treated with LPS or IL-4. F. Western blot was performed to analyze iNOS, and Arg1 expression. G. The differences in the expression of IL-12, TNF-α and Arg1, and IL-10 were measured by PCR. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Fig2

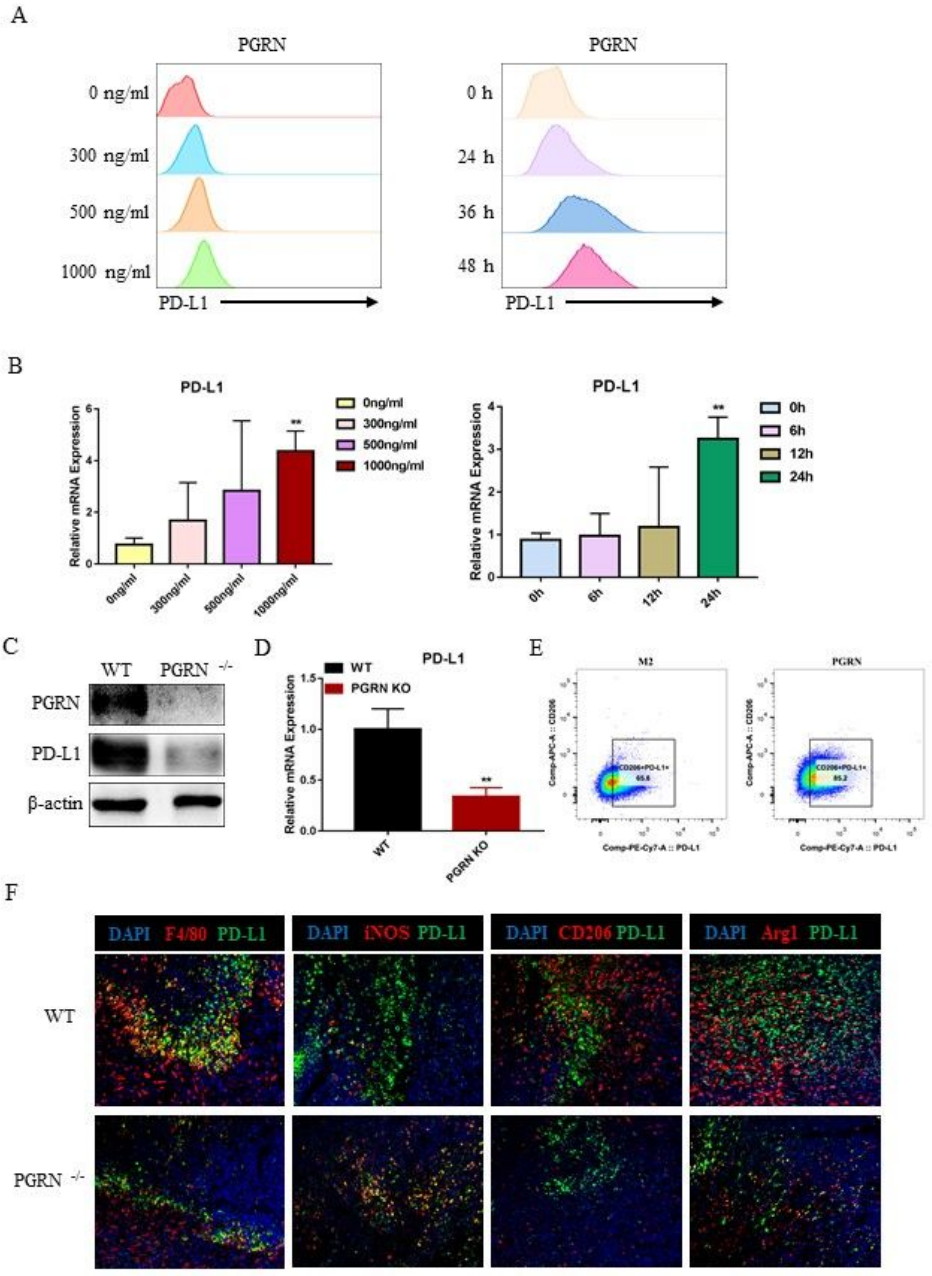

Figure 2

PGRN up-regulates PD-L1 expression on TAMs (A-B) M2 was treated with PGRN recombinant protein; then PD-L1 expression was measured by flow cytometry and PCR. (C-D). IL-4 was used to induce WT, PGRN KO peritoneal macrophages into M2; then WB and PCR were used to detect the difference in PD-L1 expression between them. E. After being treated with PGRN, the proportion of CD206+PD-L1+ cells in M2 were measured by flow cytometry. F. Immunofluorescence was performed to analyze colocalization of F4/80 (red), iNOS (red), CD206 (red), Arg1 (red) and PD-L1 (green) in WT and PGRN KO mice breast cancer sections, and the nucleus was stained with DAPI (blue). **p < 0.01.

Fig3

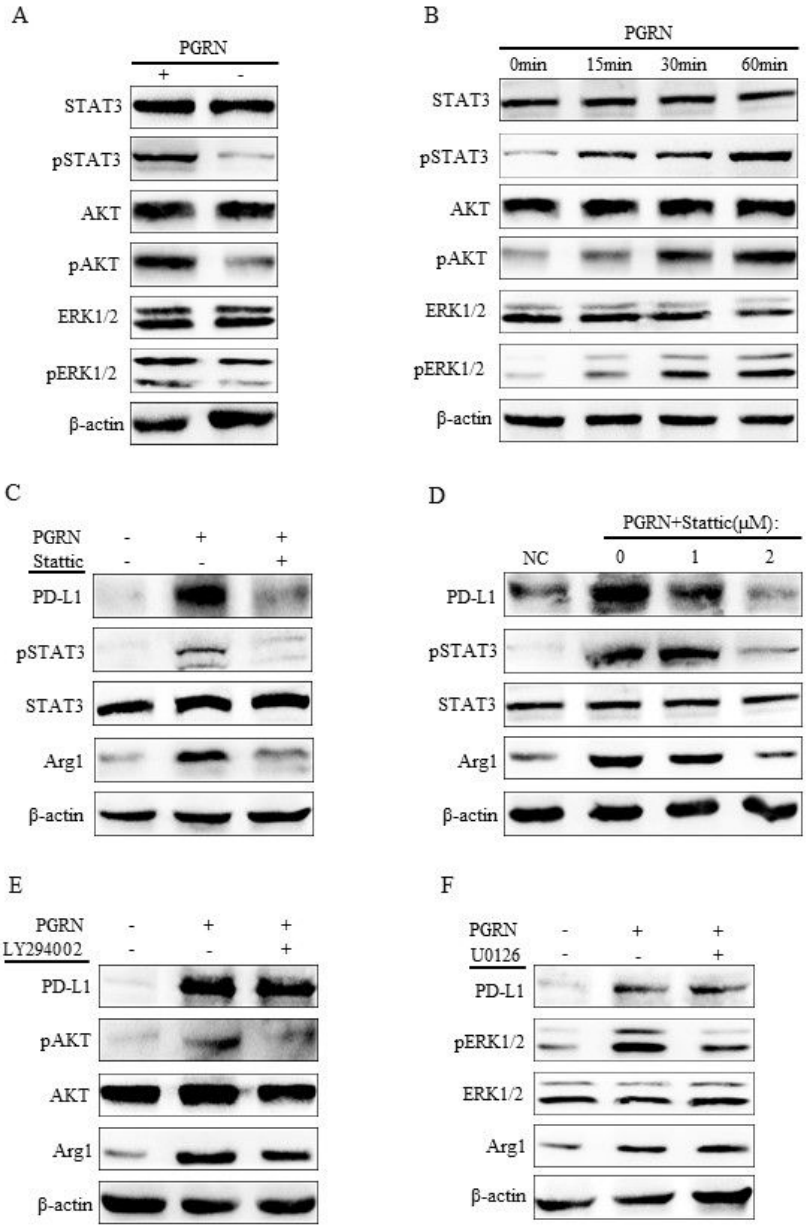

Figure 3

PGRN/STAT3 axis regulates TAMs polarization and up-regulates PD-L1 expression A. After being treated with PGRN, western blot was used to detect STAT3/pSTAT3, AKT/pAKT and ERK1/2/pERK1/2 expression in M2. B. M2 was exposed to PGRN at a specified time point, and WB was used to detect the expression of downstream signaling proteins of PGRN. (C-D). M2 was pretreated with STAT3 inhibitor Stattic, and then PGRN was added. Expression of PD-L1, STAT3/pSTAT3 and Arg1 was examined by Western blotting. (E-F) M2 was pretreated with AKT inhibitor LY294002 and ERK1/2 inhibitor U0126 respectively, and the expression changes of PD-L1, STAT3/pSTAT3 and Arg1 before and after PGRN stimulation were analyzed by Western blot.

Fig4

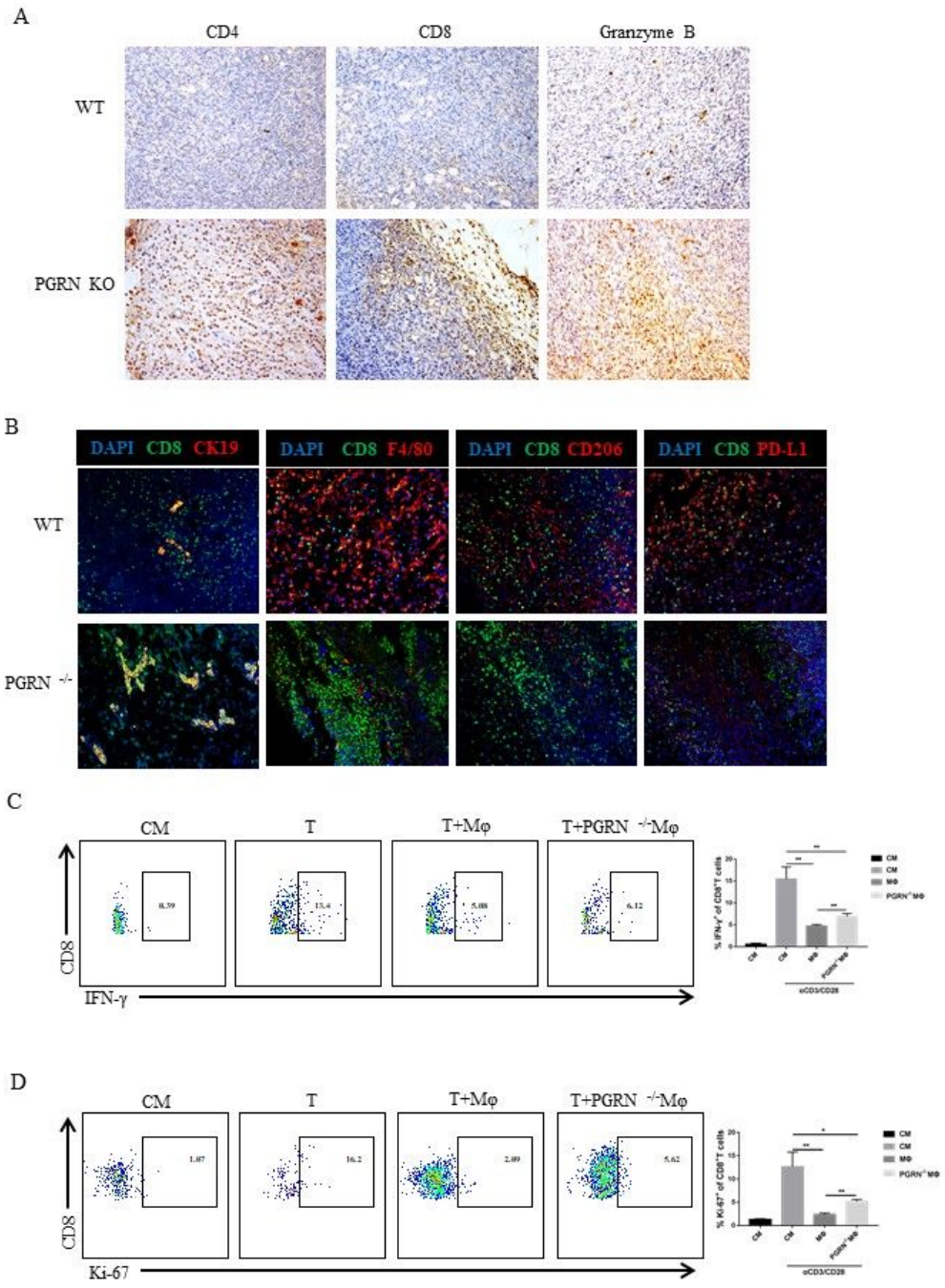

Figure 4

PGRN promotes CD8+ T cell exclusion and inhibits tumor immunity. A. The expression of CD4, CD8 and Granzyme B in WT and PGRN KO mice breast cancer tissue sections was tested by immunohistochemical staining. B. Colocalization of CK19 (red), F4/80 (red), CD206 (red), PD-L1 (red) and CD8 (green) in WT and PGRN KO mice breast cancer tissue sections was examined by immunofluorescence analysis, and the nucleus was stained with DAPI (blue). (C-D) WT and PGRN-/- peritoneal macrophages were co-

cultured with mouse spleen lymphocytes activated by αCD3/CD28. Here CM stands for control medium, without αCD3/CD28 stimulation. C. Flow cytometry was used to measure the proportion of activated CD8+T cells (IFN-γ+CD8+T cells) and (D) Ki-67+CD8+T cells. *p < 0.05; **p < 0.01.

Fig5

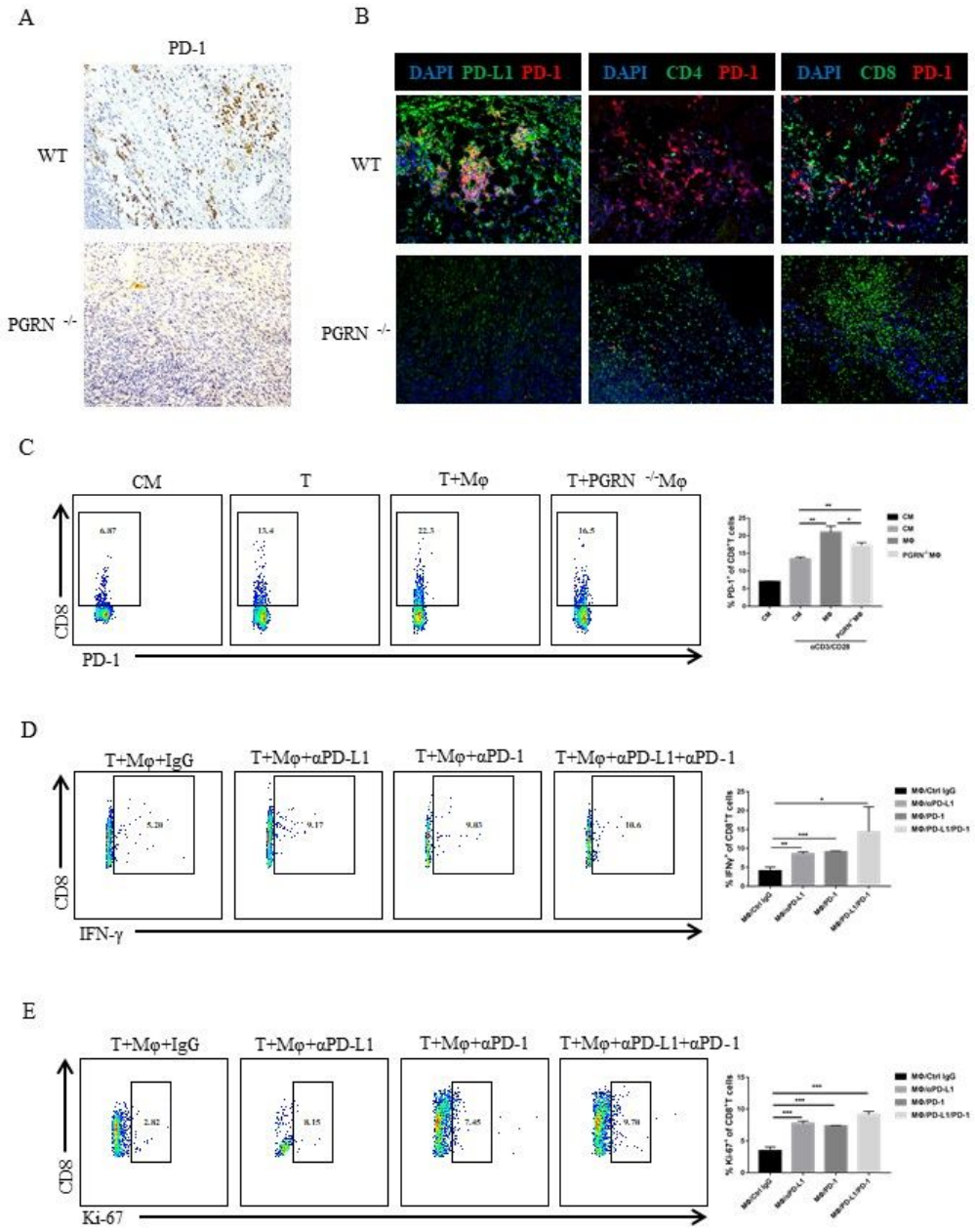

Figure 5

The interaction of PD-1/PD-L1 mediates the immunosuppressive function of PGRN in breast cancer A. Expression of PD-1 in WT and PGRN KO mice breast cancer tissue sections was detected with

immunohistochemical staining. B. PD-L1 (green), CD4 (green), CD8 (green) expression differences and co-localization with PD-1 (red) in WT and PGRN KO mice breast cancer tissue sections were examined by immunofluorescence, and the nucleus was stained with DAPI (blue). C. Mouse splenic lymphocytes activated with or without αCD3/CD28 antibody were co-cultured with WT or PGRN-/- peritoneal macrophages, and the frequency of PD-1+CD8+T cells was tested by flow cytometry. (D-E) Wild-type peritoneal macrophages were co-cultured with splenic lymphocytes preactivated by αCD3/CD28 antibody, and then anti-PD-1 or anti-PD-L1 neutralizing antibodies were added or not to the co-culture system. D. CD8+ T cell activation and (E) CD8+ T cell proliferation were detected by flow cytometry. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Supplementary Files

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