High-Baseline Tumor Burden–Associated Macrophages Promote Immunosuppressive Microenvironment and Restrain Immune Checkpoint Inhibitor Efficacy Through the IGFBP2–STAT3–PD-L1 Pathway

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Abstract

Background: In recent decades, immune checkpoint inhibitors (ICIs) have become a conspicuous promising treatment. However, there are still some problems such as limited effective rate and undefined suitable patients. With increasing attention to the influence of baseline tumor burden on the therapeutic efficacy of ICIs, most researchers are currently of the view that patients with high-baseline tumor burden (HTB) have shorter overall survival and poor immunotherapy efficacy; nevertheless, recent studies have been limited to clinical phenomena without deep mechanistic exploration.

Methods: RNA-seq and Single-cell RNA sequencing were used to reveal the difference of microenvironment between low-baseline tumor burden (LTB) and HTB tumor. Mice model construction, flow cytometry and cytokine antibody array were used to identify the phenomenon and mechanism of HTB effects on tumor immune microenvironment (TIME) and ICIs efficacy.

Results: HTB caused a significantly higher infiltration with M2-type macrophages expressing a high level of programmed death-ligand 1 (PD-L1) to block the infiltration by CD8⁺ T cells and impaired the therapeutic effect of ICIs. HTB-derived IGFBP2 induced the macrophages' polarization and PD-L1 expression via signal transducer and activator of transcription 3 (STAT3) signaling pathway. Moreover, these HTB-activated macrophages exerted a pro-tumor effect by inhibiting proliferation and cytotoxic function of CD8⁺ T cells in a PD-L1-dependent fashion. Disappointingly, total tumor burden did not affect the microenvironment or the immunotherapy efficacy of any single tumor. Moreover, macrophage inhibitor in HTB and palliative surgery were not able to increase the therapeutic efficacy in HTB. Only in the early stage of tumor with LTB, the macrophage inhibitor PLX3397 significantly improved the efficacy of ICIs on HTB.

Conclusions: Our study has demonstrated that HTB induced the production of PD-L1⁺ M2-macrophages through the IGFBP2-STAT3 signaling pathway to generate the suppressive TIME, thereby reducing the efficacy of ICIs. These results further clarify the effects and illuminate a novel mechanism of HTB effects on ICI efficacy, and also provide functional evidence for an earlier use of macrophage inhibitors or combination treatment to reshape the tumor microenvironment.

Background

In recent decades, immune checkpoint inhibitors (ICIs) have brought a new chapter in cancer treatment. Above all, therapies based on ICIs have achieved remarkable clinical outcomes and have revolutionized the treatment of various tumors, particularly of those in advanced stage. Compared with traditional cancer therapies, ICIs mainly restore the ability of effector T cells to resist tumor cells by blocking immunosuppressive receptors such as PD1/PDL1 and CTLA4; thus, ICIs have shown a wide range of anti-tumor biological activities and have achieved long-term remissions [1]. Despite the breakthrough success of ICIs, their effective rate is only 10%–30%; therefore, improving the therapeutic efficacy of ICIs is an urgent problem to be solved in clinical practice.

Effects of the location of tumor metastasis and the baseline tumor burden on the therapeutic efficacy of ICIs have also gradually received research attention. The concept of baseline tumor burden, simply defined as the size of the tumor or the total number of cancer cells before treatment, can easily be assessed by imaging. Historically, baseline tumor burden has been considered an impediment to the efficacy of ICIs [2, 3]. Patients with high-baseline tumor burden (HTB) have shorter overall survival and progression-free survival among non-small cell lung cancer (NSCLC) patients treated with atezolizumab [3]. The KEYNOTE-001 study has pointed out that HTB is associated with a lower objective response rate and decreased overall survival of patients with melanoma treated with pembrolizumab [2]. However, current research on this aspect has been limited to several clinical studies, and there is a lack of in-depth mechanistic exploration.

Tumor immune microenvironment (TIME) is another important factor that affects the efficacy of ICIs. According to the infiltration by immune cells, especially T cells, the TIME can be divided into the following three subtypes: inflamed, immune excluded, and immune desert [4]. The latter two types of tumors respond poorly to ICIs. Previous studies on the correlation between baseline tumor burden and the degree of T lymphocyte infiltration in the TIME are scarce and contradictory. The numbers of CD4⁺ and CD8⁺ T cells in peripheral blood of gastric cancer patients with low-baseline tumor burden (LTB) were higher than those of patients with HTB [5]. In diffuse large B lymphoma, there is a lower infiltration degree by Ki-67⁺ T cells in the HTB microenvironment [6]. In contrast, in ductal breast cancer, cytotoxic T lymphocytes (CTLs) infiltration was significantly increased in tumors with HTB [7]. Noteworthy, there have been no research reports that baseline tumor burden can affect tumor response to ICI therapy by influencing the TIME.

Herein, we found that HTB was able to impair the efficacy of anti-PD-1, and the number of CD8⁺ T cells was reduced in the HTB microenvironment. Single-cell sequencing revealed that activated macrophages with an immunosuppressive phenotype were highly enriched within HTB melanoma. The specific mechanism was likely as follows: HTB microenvironment prolonged the lifespan of macrophages, and released large amounts of insulin-like growth factor binding protein 2 (IGFBP2) to induce the macrophages' polarization and PD-L1 expression by activating STAT3. These PD-L1⁺ M2 macrophages inhibited CD8⁺ T cells in a PD-L1-dependent fashion, thereby reducing the response of HTB cancer to ICI therapy. To our surprise, palliative surgery was not able to improve the efficacy of ICIs. However, PLX3397-based inhibition of macrophages in the early stage of tumor effectively improved the sensitivity of HTB tumors to ICIs after tumor progression.

Methods

Patients and specimens

This study was approved by the Nanfang Hospital Ethics Review Board. A total of 51 paraffin-embedded samples from patients with colon cancer were collected. According to the medium tumour size, patients were divided into 26 LTB (average TB of 13.00 cm³) and 25 HTB (average TB of 88.11 cm³). Metastatic

tumor needle biopsy samples of 2 patients with colon cancer liver metastasis were collected. The patients were all histologically diagnosed with colon cancer at Nanfang Hospital, Southern Medical University (Guangzhou, China). We also downloaded 1 colon cancer datasets with clinical information from TCGA (COAD) and 1 melanoma datasets with clinical information from TCGA (SKCM) (<https://portal.gdc.cancer.gov/>).

Cell culture

MC38 colon cancer cell line was grown in 1640 RPMI (Gibco) supplemented with 10% FBS (Hyclone), 2 mM glutamine and 20 µg/ml gentamicin. B16 melanoma cell line was grown in high-glucose DMEM (Hyclone) supplemented with 10% FBS (Hyclone), 2 mM glutamine and 20 µg/ml gentamicin. RAW 264.7 cell line was grown in high-glucose DMEM (Hyclone) supplemented with 10% FBS (Hyclone), 2mM glutamine and 20µg/ml gentamicin. All cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. All cell lines were authenticated by the STR (Short Tandem Repeat) profiling.

Mice

C57BL/6J female mice (3-4 weeks old) from Southern Medical University Laboratory Animal Center were housed at 25 °C, and humidity was 50–60%. The light cycle consisted of light for 12 hours and dark for 12 hours.

Reagents

Anti-PD-1 antibody (10 mg/kg, BioXCell, Cat.No. BE0146), PLX3397 (40 mg/kg, Selleck, Cat.No. S7818), anti-PD-L1 (5 µg/ml, BioXCell, Cat.No. BE0101), Anti-Mouse CD45 APC (MultiSciences, Cat.No. AM04505-100), Anti-Mouse CD3 eFluor® 506 (eBioscience, Cat.No. 69-0032-82), Anti-Mouse CD8a Alexa Fluor®700 (eBioscience, Cat.No. 56-0081-82), Anti-Mouse CD11b FITC (MultiSciences, Cat.No. AH011B01-100), Anti-Mouse F4/80 PE (MultiSciences, Cat.No. AM048004-100), Anti-Mouse CD11c APC (MultiSciences, Cat.No. AM011C05-100), Anti-Mouse CD206 PE-Cyanine7 (MultiSciences, Cat.No. 25-2061-80), Anti-Mouse PD-L1 PerCP-Cy5.5 (MultiSciences, Cat.No. AM27407-20), Anti-Mouse IFN-γ APC (MultiSciences, Cat.No. AM0IF05-100), CD8 mouse monoclonal antibody (Proteintech, Cat.No. 66868-1), CD8 rabbit monoclonal antibody against mouse (Abcam, Cat.No. ab217344), ANNEXIN V-FITC/PI apoptosis detection Kit (Solarbio, Cat.No. CA1020), CFSE (Targetmol, Cat.No. T6802), Anti-Mouse Ki-67 PE (MultiSciences, Cat.No. 12-5698-80), Recombinant Mouse IL-2 (Solarbio, Cat.No. P00198), Mouse CD3 / CD28 (ThermoFisher, Cat.No. 11456D), IGFBP2 ELISA (Jiangsu Yutong, Cat.No. F5217-A), Mouse cytokine antibody array C1 (RayBiotech, Cat.No. AAM-CYT-1000-2), FLN32 (Selleck, Cat.No. 1226895-15-3), HJC0152 (Selleck, Cat.No. S8561).

Mice model construction

For LTB and HTB mice model construction. MC38 colon cancer cells or B16 melanoma cells were used to construct LTB (0.5×10^6 cells, subcutaneous growth for 14 days) and HTB (2.0×10^6 cells, subcutaneous

growth for 14 days) mice models. Mice were intraperitoneally injected with physiological saline or anti-PD-1 antibody on day 14, day 17, day 20 and day 23. Mice were sacrificed on day 26. ¶ For various TB mice model construction. MC38 colon cancer cells or B16 melanoma cells were used to construct LTB (0.5×10^6 cells, subcutaneous growth for 14 days), HTB (2.0×10^6 cells, subcutaneous growth for 14 days), SS3w (0.5×10^6 cells, subcutaneous growth for 21 days), TS1 & TS2 (TS1: 0.5×10^6 cells, subcutaneous growth for 14 days at left side. TS2: 0.5×10^6 cells, subcutaneous growth for 14 days at right side), TS2w & TS3w (TS2w: 0.5×10^6 cells, subcutaneous growth for 14 days at left side. TS3w: 0.5×10^6 cells, subcutaneous growth for 21 days at right side) mice models. Mice were sacrificed on day 21. ¶ For surgery animal model construction. MC38 colon cancer cells were injected subcutaneously on the left (2.0×10^6 cells) and right (0.5×10^6 cells) sides of the same mouse. On day 14, surgery was operated on the left subcutaneous tumor to make TB the same as that on the right side. On day 16, 18 and 21, CD3⁺CD8⁺ T cells of subcutaneous tumor were detected by flow cytometry. ¶ For drug combination mice model construction. 2.0×10^6 MC38 colon cancer cells or B16 melanoma cells were injected subcutaneously. Mice were divided into 4 groups: NC group, *PLX3397(day 7)+anti-PD-1* group, *PLX3397(day 13)+anti-PD-1* group and anti-PD-1 group. NC group without any treatment. *PLX3397(day 7)+anti-PD-1* group was treated with PLX3397 on day 7, 9, 11, *PLX3397(day 13)+anti-PD-1* group under the same treatment on day 13, 15, 17. *PLX3397(day 7)+anti-PD-1* group and *PLX3397 (day 13)+anti-PD-1* group were treated with anti-PD-1 on day 19, 22, 25, 28. *Anti-PD-1* group just under anti-PD-1 treatment on day 19, 22, 25, 28. Mice were sacrificed on day 31 for detected CD3⁺CD8⁺ T cells of subcutaneous tumor.

Immunohistochemistry (IHC) staining

IHC was performed on 4 mm sections of formalin-fixed paraffin-embedded (FFPE) tissues. Paraffin sections were baked at 65 °C for 4 hours. Sections were deparaffinized, dehydrated through a graded ethanol series. Then sections were repaired in 0.01 M and pH 6.0 citrate buffer. Endogenous peroxidase was blocked by incubation in 0.3% H₂O₂ for 10 min. After incubating with 5% BSA for 1 hour at room temperature, sections were incubated with appropriate primary antibodies overnight at 4 °C. Then sections were incubated with relevant secondary antibody for 1 hour at 37 °C. Sections were incubated with a DAB substrate kit. CD8 protein IOD were calculated by *image pro plus 6.0*.

Preparation of LTCS and HTCS and supernatant-conditioned macrophages

LTB-tumor tissue culture supernatants (LTCS) or HTB-tumor tissue culture supernatants (HTCS) were prepared by plating tumor tissues in 1 mL DMEM for 24 hours. The supernatant was then centrifuged and harvested. To generate supernatant-conditioned macrophages, RAW 264.7 were first harvested and cultured with 50% LTCS or HTCS for 24 hours, and then washed with DMEM for three times. RAW 264.7 cultured with DMEM were used as controls.

Macrophage survival assay. RAW 264.7 were stimulated with 50% LTCS or 50% HTCS, or NC for 24 hours, and then were harvested. Macrophage survival was quantified using ANNEXIN V-FITC/PI apoptosis detection Kit (Solarbio, Cat.No. CA1020).

In vitro macrophage-T cell co-culture system. In a 5-day incubation, bead-purified peripheral CD8⁺ T cells (1×10^5 cells/well in 96-well plates) were labelled with CFSE and co-cultured with macrophages (NC) or LTB MC38 subcutaneous tumour-infiltrating macrophages or HTB MC38 subcutaneous tumour-infiltrating macrophages or LTCS-conditioned or HTCS-conditioned RAW 264.7 in 200 μ L RPMI-1640 medium containing rhIL-2 (20 IU/mL), anti-CD3/CD28 (2 μ g/mL) antibodies, 2:1 for 5 days with or without anti-PD-L1 (5 μ g/ml).

Flow cytometry

Cells were resuspended to achieve a cell concentration of 1×10^7 / mL. 100 μ L cells were incubated with appropriate primary antibodies at 4 °C for 30 min. Cells were repeatedly washed twice using PBS, then cells were detected by flow cytometer. Raw data were analysed through the FlowJo_V10 software.

RNA-seq analysis of MC38 subcutaneous tumour cells

Total RNA in the tissue samples was extracted using Trizol. The mRNA was isolated and purified from the total RNA by using the Oligo (dT) magnetic beads. First-strand Illumina-barcoded libraries were generated using the NEB RNA Ultra Directional kit according to the manufacturer's instructions. Sequencing was performed by a Illumina Hiseq sequencer. Data were aligned to mouse reference genome mm10 using Bowtie2. Normalized counts and differential expression analysis was performed using the DESeq2 R package. Gene enrichment analysis was performed using the R language clusterProfiler analysis package.

Single-cell RNA sequencing

Fresh B16 melanoma subcutaneous tumor cells were washed and resuspended in 1x PBS (calcium and magnesium free) containing 0.04% BSA. 10,000 living cells were loaded on a Chromium Single Cell Controller to generate Gel Bead-In-EMulsions containing all cDNAs. Sample demultiplexing, barcode processing, alignment, filtering, UMI counting, and aggregation of sequencing runs were performed using the Cell Ranger analysis pipeline (v1.2). Seurat R package was used to merge the scRNA-seq and cell clustering and calculate the principal components analysis (PCA). Cells in which fewer than 200 genes were detected and in which mitochondrially encoded transcripts constituted more than 10% of the total library were excluded from downstream analysis. Genes detected in fewer than 3 cells across the data set were also excluded. Significant PCs were chosen through the jackstraw method. The t-SNE dimensionality reduction was generated using the RunTSNE function and the UMAP was performed with the RunUMAP function. Unsupervised clustering using a shared nearest neighbour modularity optimization based algorithm (resolution parameter 0.8) identified 16 distinct clusters.

Mouse cytokine antibody array

Cytokines from LTB and HTB mouse melanoma were detected by *AAM-CYT-1000-2 mouse cytokine antibody array*. The original data obtained from the scans were background removed and normalized

between chips using *Raybiotech software*. Differential proteins were screened using fold change (expression difference multiple) under the following selection conditions: $\geq$ fold Change $\leq$ 0.83 or fold Change $\geq$ 1.2; $\geq$ mean signal value per group > 150 .

ELISA

Subcutaneous tumors were grinded by the homogenate machine and centrifuged for 20 min at 2000 rpm. The supernatant was collected and then tested by the ELISA kit to determine the OD at 450 nm wavelength.

Statistical analyses

Graphpad Prism software (version 8.0.1) and SPSS software (version 26.0) were used for all statistical analyses. Quantitative data are shown as mean $\pm$ s.d. Statistical significance was determined using paired two-tailed Student's t-tests. Values of $p < 0.05$ were considered significant. ^{NS} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Baseline tumor burden is associated with the transformation of TIME cellular components

To explore the impact of baseline tumor burden on the TIME, we analyzed the TCGA colon cancer and melanoma data sets. Defining the T1N0M0 staging as the LTB group and the T4N0M0 staging as the HTB group, we found that the population of CD8⁺ T cells in the HTB group was less abundant than that in the LTB group, and the population of M2 macrophages showed the opposite trend in colon cancer (Figure 1A). Similar results were observed in melanoma (Figure 1B). Therefore, we speculated that baseline tumor burden is associated with the transformation of TIME cellular components.

To further verify the above results, we collected postoperative tissues from 51 colon cancer patients and metastatic tumor needle biopsy samples from 2 patients with colon cancer liver metastasis for immunohistochemistry. According to the medium tumor size, 51 colon cancer patients were divided into 26 with LTB and 25 with HTB, with an average tumor volume of 13.00 cm³ in the LTB group and 88.11 cm³ in the HTB group. Furthermore, immunohistochemical staining showed that the number of CD8⁺ T cells in HTB colon cancer patients was significantly lower than LTB (Figure 1C). Similar observations were made in metastatic tumor needle biopsy samples from patients with colon cancer liver metastasis. In this patient cohort, the population of CD8⁺ T cells in a larger metastatic tumor was less than smaller metastatic tumor in the same patient (Figure 1D). These results suggested that HTB may construct an inhibitory TIME by reducing the proportion of infiltrating CD8⁺ T cells. However, whether it affects the efficacy of ICIs through the transformation of TIME and the specific mechanism remain to be explored.

High-baseline tumor burden restricts the number of CD8⁺ T cells in mouse TIME and attenuates anti-tumor efficacy of ICIs

To define the role of baseline tumor burden on the efficacy of ICIs, we used 2×10^6 or 0.5×10^6 B16 melanoma cells or MC38 colon cancer cells to construct HTB or LTB mice models (Figure 2A and supplementary Figures 1A–B). Mice were intraperitoneally injected with physiological saline or anti-PD-1 antibody four times (Figure 2A). With anti-PD-1 treatment, the tumor growth of colon cancer in the LTB group was restrained. In contrast, the tumor growth of colon cancer in the HTB group was not ameliorated (Figures 2B–D). Similar results were observed in mice with melanoma (Figures 2E–G). To investigate the specific mechanism by which HTB weakens the efficacy of ICIs, we first evaluated the gene expression of HTB and LTB colon cancers by using RNA-Seq (Figures 2H–I). The gene-ontology (GO) enrichment analysis showed that the highly expressed genes in LTB group were those related to immune-related pathways, while those related to tumor proliferation and metastasis-related pathways dominated in the HTB group (Figure 2J). In accordance with the findings in the patient cohort, a decreased percentage of CD8⁺ T cells was observed in HTB colon cancer TIME and melanoma TIME (Figures 2K–L). Taken together, these findings suggest that HTB is associated with poor immune checkpoint inhibitor efficacy and decreased percentage of CD8⁺ T cells.

Single-cell sequencing reveals the TIME differences between LTB and HTB melanoma

Although HTB manifested an immunosuppressive TIME and a low response to immunotherapy, the underlying mechanisms remain poorly understood. Advances in single-cell RNA sequencing technologies have made it possible to comprehensively characterize the fundamental properties of tumor-infiltrating immune cells. We thereby resorted to single-cell sequencing to characterize immune features in LTB and HTB B16 melanoma showing distinct TIME and immunotherapy outcomes. After quality filtering, we obtained the single-cell transcriptome data for high-quality immune cells that were divided into 16 clusters (Figure 3A). The dot plots compare the proportion of cells expressing cluster-specific markers and their scaled relative expression levels (Figure 3B). Similarly, we found that the proportion of M2-type macrophages in the HTB group was higher (Figures 3C–D). Various studies have reported that tumor-associated macrophages (TAMs) play an important role in the CD8⁺ T cells function and immune response [8]. Such observations imply that M2-type macrophages may be responsible for the reduction of T cells and the poor efficacy of ICIs in HTB.

Macrophages mediate the formation of inhibitory TIME induced by HTB

We demonstrated that CD8⁺ T cells infiltration and the efficacy of immune checkpoint inhibitors differed between HTB and LTB constructed with different initial tumor cell numbers (Figure 2). To ensure that the effects were not specific to different initial tumor cell numbers and to exclude the physical factors of tumor size itself after rapid cell growth. Our next objective was to explore the effects of prolonged tumor growth time and increased tumor load induced by multiple lesions on the TIME and therapeutic efficacy of ICIs. We constructed the following subcutaneous tumor models: low tumor burden group (LTB, subcutaneous tumor formed on the left side with 0.5×10^6 cells for two weeks); high tumor burden group (HTB, subcutaneous tumor formed on the left side with 2×10^6 cells for two weeks); single-side subcutaneous tumor 3 weeks group (SS3w, subcutaneous tumor formed on the left side with 0.5×10^6

cells for three weeks); two-side subcutaneous tumor 2 weeks group (TS1, subcutaneous tumor formed on the left side with 0.5×10^6 cells for two weeks; TS2, subcutaneous tumor formed on the right side with 0.5×10^6 cells for two weeks); two-side subcutaneous tumor 2 weeks and 3 weeks groups (TS2w, subcutaneous tumor formed on the left side with 0.5×10^6 cells for two weeks; TS3w, subcutaneous tumor formed on the right side with 0.5×10^6 cells for three weeks) (Figure 4A and supplementary Figure 2A).

Consistent with Figure 2K, in the MC38 colon cancer subcutaneous tumor mice model, the number of CD8⁺ T cells infiltrating TIME in the HTB group was significantly lower than that in the LTB group (Figure 4B, Top). We also found that the number of CD8⁺ T cells in TIME in the SS3w group was lower than that in the LTB group (Figure 4B, Top). These data indicated that the quantity of CD8⁺ T cells in the TIME decreased with the tumor growth, showing that HTB blocked the infiltration of CD8⁺ T cells independent of the physical factor of tumor size. Surprisingly, the number of CD8⁺ T cells in TIME of the TS1 and TS2 groups was similar to that in the LTB group, and was not reduced due to the augmentation in the total tumor burden (Figure 4B, Middle). Furthermore, the number of CD8⁺ T cells in the TIME of the TS2w group was similar to that in the LTB group and TS1/2 group, but higher than that in the TS3w group (Figure 4B, Bottom). The above findings illustrate that the counts of CD8⁺ T cells in TIME are only related to single tumor burden, and are independent of the initial tumor cell number and the total tumor burden. Next, we found the same situation in the B16 melanoma subcutaneous tumor mice model (Figure 4C). In addition, we intended to examine whether reducing the tumor burden could reform the immunosuppressive microenvironment of HTB tumors. To that end, we constructed a debulking surgery model using MC38 colon cancer cells: subcutaneous tumor formed on the left side with 2×10^6 cells for two weeks as HTB cancer and subcutaneous tumor formed on the right side with 0.5×10^6 cells for two weeks as LTB cancer. We reduced the tumor on the left side to the same tumor burden as that on the right side on day 14, and then detected the quantity of CD8⁺ T cells in TIME on days 16, 18, and 21 (Figure 4D). Disappointingly, we did not find that reducing the tumor size of HTB mice was able to reform the immunosuppressive microenvironment of HTB tumors (Figure 4E–G).

These findings together imply that HTB promotes the formation of immunosuppressive microenvironment by reducing the number of CD8⁺ T cells as the tumor grows in the TIME. To understand the reason for this phenomenon, we conducted single-cell sequencing. Our results showed that there were significant differences in M2-type macrophages between the HTB and LTB groups; specifically, the proportion of M2-type macrophages in the HTB group was significantly higher than that in the LTB group. Therefore, we hypothesized that M2-type macrophages in the HTB group affected the infiltration by CD8⁺ T cells and the anti-tumor efficacy of PD-1 blockade. To test this hypothesis, we used 2×10^6 melanoma cells or MC38 colon cancer cells to construct therapeutic mice models. Mice with different tumor burden were intragastrically administered with or without the macrophage inhibitor PLX3397 (Figure 4H). Using PLX3397 on day 7 or day 13 did not affect the tumor burden on day 19 (supplementary Figure 2B). Compared with the group that only received PD-1 inhibitor, the group using PLX3397 at HTB (on day 13) and PD-1 inhibitor (on day 19) neither showed a significant delay in the

tumor growth (Figure 4I), nor promoted the infiltration of colon cancer TIME by CD8⁺ T cells (Figure 4K). Interestingly, the group receiving PLX3397 at an early stage of the tumor (day 9) and PD-1 inhibitor on day 19 displayed a powerful delay in tumor growth (Figure 4I) and augmented proportion of CD8⁺ T cells in the TIME (Figure 4J). Similar results were observed in melanoma mice models (Figure 4K, L). These findings indicate that abundant M2-type macrophages in HTB result in reduced number of CD8⁺ T cells and poor immunotherapy effect. Moreover, the timing of the inhibition of macrophages was incredibly important for [remodeling](#) immunosuppressive microenvironment and improving the efficacy of PD-1 inhibitors for HTB tumors. Using PLX3397 at HTB could not restore the number of CTLs in the TIME because the number of CTLs in the HTB microenvironment had already been reduced.

HTB-TIME promotes the formation of PD-L1⁺ M2 macrophages

The above results indicated that HTB environments incur the aggregation of M2-type macrophages. Thus, we next examined the possible mechanisms. We speculated that HTB colon cancer and HTB melanoma microenvironment sustained the survival of macrophages. To verify this speculation, we tested the survival of macrophages after exposure to colon cancer tissue culture supernatants (TCS) or melanoma TCS. The results showed that macrophages exposed to HTB tissue culture supernatants (HTCS) exhibited a delayed onset of apoptosis when compared with those exposed to LTB tissue culture supernatants (LTCS) and control culture medium (NC) (Figures 5A–B). Furthermore, compared with the other two groups, macrophages exposed to HTCS showed a higher proportion of M2 type (Figures 5C–D).

Simultaneously, we also speculated that HTB environments contribute to the activated immunosuppressive phenotype of macrophages. As expected, compared with LTCS or NC-conditioned macrophages, the co-expression of CD206 and PD-L1 on HTCS-conditioned macrophages was upregulated significantly (Figures 5E–H). These results indicate that HTB colon cancer and melanoma microenvironments promote the survival of macrophages and enhance the expression of PD-L1 on M2 macrophages.

HTB-associated PD-L1⁺ M2 macrophages inhibit T cells through the PD-1/PD-L1 axis

M2 macrophages can exert inhibitory effects on T cells in various ways, including secretion of inhibitory factors, metabolic competition, or expression of inhibitory effector molecules [9-11]. In view of the high PD-L1 expression on HTB-associated macrophages, we next investigated the impact of PD-L1 on HTB-associated macrophages in T cell suppression by adding neutralizing antibodies against PD-L1 into a macrophage/peripheral T cell co-culture system. First, purified peripheral CD8⁺ T cells were co-cultured with tumor-infiltrating macrophages or TCS-conditioned macrophages. HTB tumor-infiltrating macrophages with high expression CD206 and PD-L1 significantly suppressed the proliferation of CD8⁺ T cells (Figures 5A–B). Then, we added neutralizing antibodies against PD-L1 to the macrophages/T cells co-culture system. As expected, PD-L1 neutralizing antibody weakened the suppression of T cells' survival by HTB tumor-infiltrating macrophages (Figures 5A–B). HTCS-conditioned macrophages showed the same effects (Figures 5C–D). Blocking PD-L1 was also able to restore the IFN- γ production by T cells,

which had been inhibited by HTCS-conditioned macrophages (Figures 5C–D). These findings show that tumor-infiltrating macrophages that are activated by HTB-TIME suppress T cell proliferation and cytotoxicity through the PD-1/PD-L1 axis.

HTB induces PD-L1⁺ M2 macrophages by the IGFBP2–STAT3 axis

Tumor microenvironment contains a variety of soluble factors, including cytokines, inflammatory factors, and chemokines, all of which play an important role in cell–cell interaction, reshaping the tumor microenvironment and affecting the therapeutic efficacy [12]. To detect which cytokines activate and induce PD-L1 expression on macrophages, we first screened cytokines in mouse melanoma environments by a mouse cytokines' antibody array. Compared with LTB, pro-MMP-9, bFGF, MMP-2, IGFBP-2, TIMP-2, MCSF, PF4, and other cytokines were highly expressed in the HTB microenvironment; among them, the highly expressed cytokine was IGFBP2 (Figure 7A). In addition, the content of IGFBP2 in melanoma HTCS was significantly higher than that in LTCS as detected by ELISA (Figure 7B), and only IGFBP2 remarkably induced mononuclear macrophages to PD-L1⁺ M2 macrophages (Figures 7C–D and supplementary Figures 3A–B). Next, we examined the effect of macrophages activated by IGFBP2 on CD8⁺ T cells. Macrophages activated by IGFBP2 for 24 hours were co-cultured with CD8⁺ T cells. In the meantime, we added neutralizing antibodies or phosphate buffer saline (PBS) to the IGFBP2-induced macrophages/T cells co-culture system. The results showed that IGFBP2-induced macrophages effectively suppressed CD8⁺ T cells proliferation, and blockade of PD-L1 efficiently revoked T cell suppression mediated by such macrophages (Figure 7E). Based on this result, we may conclude that HTB mainly promotes the formation of immunosuppressive TIME through PD-L1⁺ M2 macrophages induced by IGFBP2. However, previous studies on the effect of IGFBP2 on macrophages are limited. Only one study reported that IGFBP2 could activate STAT3 to construct an immunosuppressive tumor microenvironment infiltrated with M2-type macrophages in pancreatic duct cancer [13]. Interestingly, we found that abolishing the phosphorylation of STAT3 with an inhibitor (FLLL32 or HJC0152) effectively suppressed the formation of PD-L1⁺ M2 macrophages induced by IGFBP2 or HTCS (Figures 7F–G and supplementary Figure 3C). These results suggest that HTB-TIME induces macrophages with immunosuppressive phenotype mainly through the IGFBP2–STAT3 pathway.

Discussion

Although ICIs therapy can tremendously improve the survival of patients with various cancers, the proportion of patients who benefit from ICIs is only 10%–30%, and some treated patients have serious immune-related adverse events. Multiple clinical studies have shown that baseline tumor burden is significantly associated with poor prognosis and treatment outcomes after immunotherapy [14–16]. Patients with HTB tend to have poorer immunotherapy response and survival outcomes. However, research on the tumor baseline burden and immunotherapy efficacy has been limited to clinical studies, and the specific mechanism remains unclear. In this study, we showed that immunotherapies of HTB colorectal cancer and melanoma were constrained, and that the number of infiltrating CD8⁺ T cells in the

microenvironment was reduced, which indirectly suggests that HTB promotes the formation of immune desert TIME to diminish the efficacy of ICIs.

A question often arises as to whether palliative surgery can increase the efficacy of subsequent immunotherapy in patients with advanced cancer, and whether patients with early-stage tumors have better immunotherapy efficacy than those with advanced-stage tumors [17]. Through the comprehensive animal models, we solved the above questions and obtained other major findings as follows: 1) The number of CD8⁺ T cells and M2-type macrophages in the TIME of tumor-bearing mice showed time-dependent changes. To rule out that the tumor logarithmic growth of HTB physically prevented CD8⁺ T cell infiltration, we designed a tumor-bearing mouse model with the same initial number of tumor cells at different growth times. We found that it was not the tumor size that blocked the CD8⁺ T infiltration; rather, with tumor growth, the abundance of M2-type macrophages gradually increased, while CD8⁺ T cells showed the opposite trend in a time-dependent manner. 2) Total tumor burden did not affect the microenvironment or the immunotherapy efficacy of a single tumor, which indirectly indicated that metastatic tumors might not affect the efficacy of immunotherapy in the primary tumor. 3) Reducing the tumor size of HTB mice by surgery did not effectively augment the number of CTLs in the TIME and boost immunotherapy efficacy, which further indicates that the effect on TIME transformation and the therapeutic effect of HTB are independent of physical factors, which may be due to the fact that the desert microenvironment caused by M2-type macrophages has not been ameliorated. Some researchers believe that reducing the tumor size by palliative operation, chemotherapy, and radiotherapy should be explored as a way to improve the efficacy of ICIs in HTB cancer patients. Disappointingly, in our study, we demonstrated that the efficacy of immunotherapy after palliative surgery in colon cancer or melanoma patients with advanced tumors was not improved. However, previous studies have suggested that chemoradiation may induce T-cell activation by producing new mutations [18, 19], which is a potential entry point for improving the immune benefit of HTB in the future.

This study showed that the effect of HTB on the efficacy of immunotherapy was due to the immunosuppressive microenvironment constructed by HTB-related macrophages. Macrophages in the TIME are mainly divided into M1 and M2 types. M2 macrophages can exert inhibitory effects on T cells by secreting inhibitory factors, performing metabolic competition, expressing inhibitory effector molecules, and inducing stromal fibrosis to expel T cells [9-11, 20]. Previous studies have demonstrated that endoplasmic reticulum stress, oxidative stress, fatty acid oxidation, lactate, and matrix stiffness can drive macrophage M2-polarization [21-24]. Our research innovatively pointed out that HTB may also cause macrophages to switch to M2-type and reduce the apoptosis of macrophages in the TIME. In addition, HTB-associated macrophages inhibited CD8⁺ T cells through the PD-1/PD-L1 axis; in the macrophage/peripheral T cell co-culture system, anti-PD-L1 antibody effectively decreased the inhibitory effect of HTB-activated macrophages on CTLs. Although HTB-associated macrophages inhibit T cells through the PD-1/PD-L1 axis, immunotherapy is still ineffective in HTB, which may be because CTLs have already been reduced in the HTB microenvironment. Next, we explored the efficacy of a combination regimen of macrophage inhibitors and immunotherapy. Notably, macrophage colony-stimulating factor-1

(CSF-1) inhibitor itself did not affect the tumor growth, but the timing of using CSF-1 inhibitor was crucial; namely, only using CSF-1 inhibitor when the tumor burden is low was able to significantly strengthen the efficacy of anti-PD-1 in HTB mice. This conclusion further defines the immune desert TIME in HTB; at that time, inhibiting M2 macrophages with a CSF-1 inhibitor cannot restore the number of CTLs in the tumor microenvironment. The above results suggest that the combination of CSF-1 inhibitor and ICIs cannot bring significant benefit to patients with advanced-stage tumors; nevertheless, earlier application of CSF-1 inhibitor to ameliorate the tumor microenvironment could improve the efficacy of second-line or even post-line immunotherapy treatments for patients. In summary, HTB weakened the efficacy of immunotherapy not due to the physical factor of tumor size, but due to the desert immune microenvironment constructed by HTB-associated macrophages. Therefore, debulking surgery and macrophage inhibitors used in HTB could not change the outcome of poor immunotherapy efficacy in HTB.

Next, we investigated the specific mechanism of HTB-associated macrophages production. By isolating and obtaining the culture supernatant of tumor tissue, we found that HTB regulates TAM through the secretory pathway. Tumor cells can secrete a variety of soluble factors, including cytokines, inflammatory factors, and chemokines; in that way, they can reshape the tumor microenvironment and affect tumor progression and therapeutic efficacy [12]. In this study, we found that compared with LTB, HTB secreted large quantities of cytokines, including pro-MMP-9, β FGF, MMP-2, IGFBP-2, TIMP-2, MCSF, and PF4. Among them, only IGFBP2 was able to promote the polarization of macrophages and induce the PD-L1 expression, which were also first demonstrated in HTB tumors. IGFBP2 was identified as a regulator of the IGF system; it plays a significant role in several crucial oncogenic processes, such as cancer cells migration, invasion, proliferation, vascular formation, and epithelial–mesenchymal transition, by regulating various signal transductions [25-28]. Previous studies about the effect of IGFBP2 on macrophages are limited. We confirmed that IGFBP2 not only promoted macrophage polarization but also regulated PD-L1 transcription through the STAT3 signaling pathway, which is consistent with the results of Zhang and Yang [13, 29]. STAT3, a member of the signal and activator of transcription (STAT) protein family, is involved in a variety of biological processes, including cell growth, differentiation, and angiogenesis [30, 31]. Anomalous activated STAT3 in tumor cells is associated with poor prognosis [32]. Accumulating evidence has revealed that STAT3 participates in regulating the formation of immunosuppressive microenvironment by inhibiting the release of proinflammatory cytokines, damaging antigen presentation, and inhibiting the aggregation and cell killing function of effector cells [33-35]. In addition, as a transcription factor, STAT3 can also directly promote the transcription of PD-L1 and PD-L2 [36, 37]. Thus, the combination therapy targeting STAT3 is considered a promising direction for improving immunotherapy efficacy. Various STAT3 inhibitors in combination with immune checkpoint blockers are currently being tested in clinical trials; among them, STAT3 inhibitor BBI608 was approved by FDA for gastric and pancreatic cancer based on the promising results of phase I/II clinical trials. The phase II trials of BBI608, in combination with Pembrolizumab (NCT03647839) or nivolumab (NCT02851004), in metastatic colorectal cancer are ongoing. Combined with the conclusions of this study, STAT3 inhibitors might be used as sensitizers for HTB immunotherapy, and their application in first-line may improve the

clinical efficacy of second-line or even post-line immunotherapy. However, how IGFBP2 activates STAT3 and the specific mechanism of the different levels of IGFBP2 secreted by tumor cells with HTB and LTB have not been clarified. The large amount of IGFBP2 in HTB may be related to hypoxia or nutrient deficiency in HTB TME [38], which needs to be further investigated.

Conclusion

In summary, we defined the effects of baseline tumor burden on the TIME and immunotherapy efficacy. HTB induced the production of PD-L1⁺ M2 macrophages via the IGFBP2–STAT3–PD-L1 pathway to promote the generation of immunosuppressive TIME (Figure 8). Our results provide a better understanding of the role of HTB on ICIs efficacy, and a functional evidence for an earlier use of macrophage inhibitors or combination treatment to against tumor.

Abbreviations

ICIs	immune checkpoint inhibitors
HTB	high-baseline tumor burden
PD-L1	programmed death-ligand 1
STAT3	signal transducer and activator of transcription 3
TIME	tumor immune microenvironment
LTB	low-baseline tumor burden
NSCLC	non-small cell lung cancer
CTLs	cytotoxic T lymphocytes
IGFBP2	insulin-like growth factor binding protein 2
TAMs	tumor-associated macrophages
TCS	tissue culture supernatants
HTCS	HTB tissue culture supernatants
LTCS	LTB tissue culture supernatants
PBS	phosphate buffer saline
CSF-1	macrophage colony-stimulating factor-1

Declarations

Ethics approval and consent to participate

This study was approved by the Human Research Ethics Committee of Nanfang Hospital.

Consent for publication

Not applicable.

Availability of data and materials

All data and materials are available to the researchers once published.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WZW, RZ, HYS and WJL contributed to the planning of the study and drafted the manuscript. WJL contributed to manuscript revision. WZW, RZ, HYS and ZHZ performed all the experiments, prepared all the figures and tables. STZ, JPB, YLL and MS contributed to interpretation of data, and review of the manuscript. All the authors reviewed and approved the final manuscript.

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Figures

Figure 1

HTB is associated with the decreased amount of CD8⁺ T cells in TIME.

(A) TCGA colon cancer and (B) melanoma datasets. Compared with LTB group (T1N0M0), the population of CD8⁺ T cells decreased (A. $p < 0.05$ and B. $p < 0.05$) and the population of M2 macrophages increased (A. $p < 0.01$ and B. $p < 0.005$) in the HTB group (T4N0M0). (C) CD8⁺ T cells in postoperative tissues from 51 colon cancer patients. According to the medium tumour size, patients were divided into 26 LTB (average TB of 13.00 cm³) and 25 HTB (average TB of 88.11 cm³). The number of CD8⁺ T cells in HTB group significantly decreased ($p < 0.001$, scale bar=100 μ m). (D) CD8⁺ T cells in metastatic tumour needle biopsy samples. The population of CD8⁺ T cells in the larger metastatic tumor is reduced (n=2, $p < 0.001$, scale bar=100 μ m).

Figure 2

HTB is associated with the decreased amount of CD8⁺ T cells in TIME and the attenuated efficacy of ICB.

(A) Schematic diagram of LTB & HTB animal model construction. Used MC38 cells or B16 melanoma cells to construct LTB (0.5×10^6 cells, subcutaneous growth for 14 days) and HTB (2.0×10^6 cells, subcutaneous growth for 14 days) mice models. Mice were injected with physiological saline or anti-PD-1 antibody (10 mg/kg) on day 14, day 17, day 20 and day 23. Mice were sacrificed on day 26. (B) Tumor

size, (C) tumor growth rate and (D) tumor final size of MC38 subcutaneous tumor ($n=5$ per group, $^{NS}p > 0.05$, $^{***}p < 0.001$). (E) Tumor size, (F) tumor growth rate and (G) tumor final size of B16 subcutaneous tumor ($n=5$ per group, $^{NS}p > 0.05$, $^{***}p < 0.001$). (H) Volcano plot and (I) heatmap showed the difference gene expression of HTB and LTB colon cancers ($n=5$ per group). (J) GO Enrichment analysis showed that the high expression genes in LTB and HTB MC38 subcutaneous tumor. (K) CD8⁺ T cells in MC38 subcutaneous tumor ($^{***}p < 0.001$, scale bar=100 μ m). (L) CD8⁺ T cells in B16 subcutaneous tumor ($^{***}p < 0.001$, scale bar=100 μ m).

Figure 3

Single-cell sequencing reveals the TIME differences between LTB and HTB melanoma.

(A) TIME cellular components were divided into 16 clusters. (B) Clusterspecific markers of each clusters. (C) M1 and M2 macrophages in LTB and HTB TIME. (D) Proportions of M1 and M2 macrophages in LTB and HTB TIME.

Figure 4

HTB induced the inhibitory TIME through macrophages.

(A) Schematic diagram of various TB mice model construction. MC38 cells or B16 melanoma cells were used to construct LTB (0.5×10^6 cells, subcutaneous growth for 14 days), HTB (2.0×10^6 cells, subcutaneous growth for 14 days), SS3w (0.5×10^6 cells, subcutaneous growth for 21 days), TS1 & TS2 (TS1: 0.5×10^6 cells, subcutaneous growth for 14 days at left side. TS2: 0.5×10^6 cells, subcutaneous growth for 14 days at right side), TS2w & TS3w (TS2w: 0.5×10^6 cells, subcutaneous growth for 14 days at left side. TS3w: 0.5×10^6 cells, subcutaneous growth for 21 days at right side) mice models. (B) CD3⁺CD8⁺ T cells in MC38 subcutaneous tumor ($n=3$ per group, $^{NS}p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (C) CD3⁺CD8⁺ T cells in B16 subcutaneous tumor ($n=3$ per group, $^{NS}p > 0.05$, $^{***}p < 0.001$). (D) Schematic diagram of surgery mice model construction. MC38 cells were injected subcutaneously on the left (2.0×10^6 cells) and right (0.5×10^6 cells) sides of the same mouse. In order to make the tumor volume on both sides equal, tumor reduction surgery was performed on the left subcutaneous tumor on day 14. (E) On day 16, (F) day 18 and (G) day 21, CD3⁺CD8⁺ T cells in left side or right side subcutaneous tumor were detected by flow cytometry ($n=3$ per group, $^{NS}p > 0.05$, $^{**}p < 0.01$). (H) Schematic diagram of mice model treated with PLX3397 (40 mg/kg) and anti-PD-1 (10 mg/kg). Initially, 2.0×10^6 MC38 cells or B16 cells were injected subcutaneously. One group was treated with PLX3397 on day 7, 9, 11 (top), the other group under the same treatment on day 13, 15, 17 (bottom). Both of them were treated with anti-PD-1 on day 19, 22, 25, 28, then mice were sacrificed on day 31 for detected CD3⁺CD8⁺ T cells in subcutaneous tumor. NC

group without any treatment. Anti-PD-1 group just under anti-PD-1 treatment on day 19, 22, 25, 28. (I) Tumor volume of 4 group MC38 mice models ($n=3$ per group, $^{NS}p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (J) $CD3^+CD8^+$ T cells in MC 38 subcutaneous tumor ($^*p < 0.05$, $^{**}p < 0.01$). (K) Tumor volume of 4 group B16 mice models ($n=3$ per group, $^{NS}p > 0.05$, $^{**}p < 0.01$). (L) $CD3^+CD8^+$ T cells in B16 subcutaneous tumor ($^*p < 0.05$, $^{**}p < 0.01$).

Figure 5

HTB TIME promoted the formation of PD-L1⁺ M2 macrophages.

(A) Proportion of apoptotic macrophages after exposure to serum free medium (NC), MC38 low tumor burden subcutaneous tumor tissue culture supernatants (LTCS) and MC38 high tumor burden subcutaneous tumor tissue culture supernatants (HTCS) for 24 hours ($n=3$ per group, $^*p < 0.05$, $^{***}p < 0.001$). (B) Proportion of apoptotic macrophages after exposure to B16 subcutaneous tumor tissue culture supernatants ($n=3$ per group, $^*p < 0.05$, $^{**}p < 0.01$). (C) Proportion of M2 macrophages after exposed to MC38 subcutaneous tumor tissue culture supernatants ($n=3$ per group, $^{NS}p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$). (D) Proportion of M2 macrophages after exposed to B16 subcutaneous tumor tissue culture supernatants ($n=3$ per group, $^{NS}p > 0.05$, $^*p < 0.05$, $^{***}p < 0.001$). (E) Proportion of PD-L1⁺ M2 macrophages after exposed to MC38 subcutaneous tumor tissue culture supernatants ($n=3$ per group,

^{NS} $p > 0.05$, ^{***} $p < 0.001$). (F) Expression of CD206 or PD-L1 of macrophages after exposed to MC38 subcutaneous tumor tissue culture supernatants (n=3 per group, ^{NS} $p > 0.05$, ^{***} $p < 0.001$). (G) Proportion of PD-L1⁺ M2 macrophages after exposed to B16 subcutaneous tumor tissue culture supernatants (n=3 per group, ^{NS} $p > 0.05$, ^{***} $p < 0.001$). (H) Expression of CD206 or PD-L1 of macrophages after exposed to B16 subcutaneous tumor tissue culture supernatants (n=3 per group, ^{NS} $p > 0.05$, ^{*} $p < 0.05$, ^{**} $p < 0.01$).

Figure 6

HTB-associated PD-L1⁺ M2 macrophages inhibited T cells through PD-1/PD-L1 axis.

(A) Purified peripheral CD8⁺ T cells were co-cultured with macrophages (NC) or LTB MC38 subcutaneous tumour-infiltrating macrophages or HTB MC38 subcutaneous tumour-infiltrating macrophages 2:1 for 5 days with or without anti-PD-L1 (5 µg/ml). Ki-67⁺ CD8⁺ T cells were detected by flow cytometry (n=3 per group, ^{*} $p < 0.05$, ^{**} $p < 0.01$). (B) Purified peripheral CD8⁺ T cells were co-cultured with macrophages (NC) or LTB B16 subcutaneous tumour-infiltrating macrophages or HTB MC38 subcutaneous tumour-infiltrating macrophages 2:1 for 5 days with or without anti-PD-L1 (5 µg/ml) Ki-67⁺CD8⁺ T cells were detected by flow cytometry (n=3 per group, ^{NS} $p > 0.05$, ^{**} $p < 0.01$). (C) Macrophages were exposed to cell culture medium or MC38 LTCS or HTCS for 24 hours. Purified peripheral CD8⁺ T cells were co-cultured with macrophages (NC) or LTCS-associated macrophages or HTCS-associated macrophages 2:1 for 5 days with or without anti-PD-L1 (5 µg/ml). Proliferated CD8⁺ T cells or proliferated IFNγ⁺CD8⁺ T cells were detected by flow cytometry (n=3 per group, ^{NS} $p > 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$). (D) Macrophages were exposed to cell culture medium or B16 LTCS or HTCS for 24 hours. Purified peripheral CD8⁺ T cells were co-cultured with macrophages (NC) or LTCS-associated macrophages or HTCS-associated macrophages for 5 days with or without anti-PD-L1 (5 µg/ml). Proliferated CD8⁺ T cells or proliferated IFNγ⁺CD8⁺ T cells were detected by flow cytometry (n=3 per group, ^{NS} $p > 0.05$, ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$).

Figure 7

HTB induced PD-L1⁺ M2 macrophages by IGFBP2-STAT3 axis.

(A) Mouse cytokines antibody array displayed the cytokines content between mouse LTB and HTB melanoma environments. (B) ELISA detected IGFBP2 content between mouse LTB and HTB melanoma environments (n=3 per group, ^{**} $p < 0.01$). (C) Macrophages were exposed to cell culture medium or IGFBP2 (10 ng/ml) for 24 hours. PD-L1⁺ M2 macrophages were detected by flow cytometry (n=3 per group, ^{***} $p < 0.001$). (D) Expression of CD206 or PD-L1 of macrophages after exposed to cell culture medium or IGFBP2 (10 ng/ml) for 24 hours (n=3 per group, ^{*} $p < 0.05$, ^{**} $p < 0.01$). (E) Macrophages were exposed

to cell culture medium or IGFBP2 (10 ng/ml) for 24 hours. Purified peripheral CD8⁺ T cells were co-cultured with macrophages (NC) or IGFBP2-associated macrophages 2:1 for 24 hours with or without anti-PD-L1 (5 µg/ml) (n=3 per group, ** $p < 0.01$, *** $p < 0.001$). (F) Macrophages were exposed to cell culture medium, IGFBP2 (10 ng/ml), IGFBP2 & HJC0152 (5 µM) or IGFBP2 & FLLL32 (5 µM) for 24 hours. PD-L1⁺ M2 macrophages were detected by flow cytometry (n=3 per group, ** $p < 0.01$, *** $p < 0.001$). (G) Expression of CD206 or PD-L1 of macrophages after exposed to cell culture medium, IGFBP2 (10 ng/ml), IGFBP2 & HJC0152 (5 µM) or IGFBP2 & FLLL32 (5 µM) for 24 hours (** $p < 0.01$, *** $p < 0.001$).

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