



Trained immunity alleviates the progression of melanoma during sepsis-associated immunoparalysis

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Abstract

Background Patients who survive the excessive inflammatory phase of sepsis experience prolonged immunoparalysis/immunosuppression. During this phase, the patient's immune system is severely impaired, which increases the patient's susceptibility to septic complications. Sepsis survivors have a significantly greater incidence of cancer, but the mechanism underlying this phenomenon is unknown.

Methods We constructed two sepsis–melanoma models to assess the relationship between sepsis and sepsis-related concomitant cancer. In our investigation, we employed a range of experimental technique to elucidate the intricate mechanisms through which the immunoparalysis phase of sepsis facilitates melanoma progression. Furthermore, we induced trained immunity with oroxylin A (OA) to evaluate its ability to reverse immunoparalysis and subsequent tumor progression in sepsis-melanoma models.

Results We showed that sepsis upregulated the serum level of interleukin (IL)-6 and the number of myeloid-derived suppressor cells (MDSCs), regulated G-MDSCs/M-MDSCs and inhibited CD8⁺T-cell function, which promoted melanoma progression. OA-induced trained immunity can reverse immunoparalysis, maintain the antitumor capacity of the immune system, and inhibit the development of sepsis-complicated melanoma. Notably, OA can target macrophage migration inhibitory factor (MIF) and downregulate the serum level of IL-6, which may be a crucial molecular mechanism by which OA induces trained immunity to reverse the immunoparalysis phase of sepsis.

Conclusion Sepsis can promote cancer progression by upregulating MIF and IL-6, increasing the G-MDSCs/M-MDSCs ratio and reducing the number and function of CD8⁺ T cells, leading to immunoparalysis, while trained immunity can alleviate this progression. The findings of this study provide new strategies for preventing or treating sepsis-complicated cancer.

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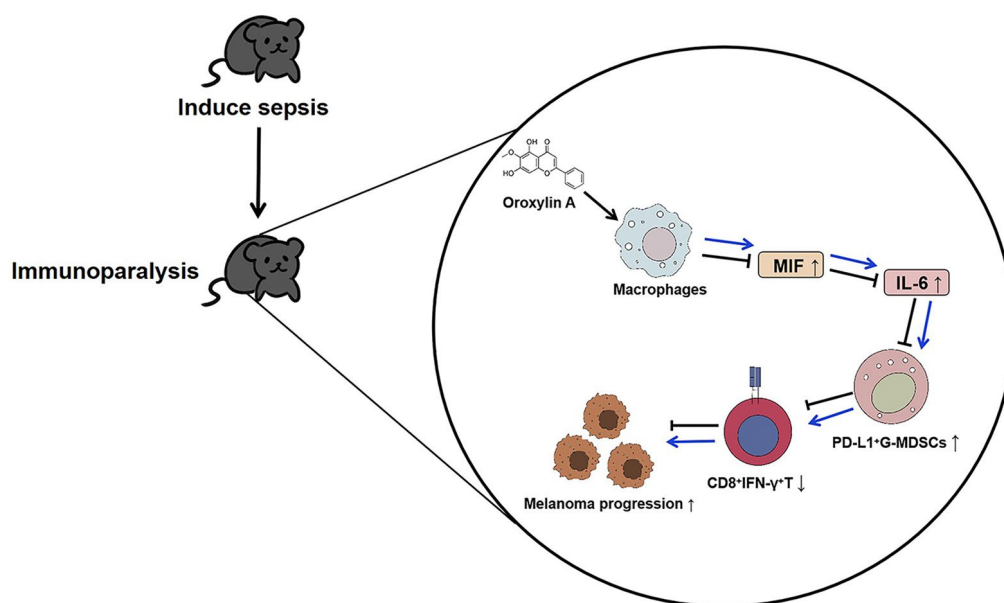
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Graphical Abstract



Keywords Sepsis · Immunoparalysis · Melanoma · Trained immunity · Oroxylin A

1 Introduction

Sepsis is a clinical syndrome characterized by systemic multiorgan failure due to a dysregulated host response to infection [1]. In the early stage of sepsis, the main cause of patient death is excessive inflammation caused by cytokine storms, with a mortality rate of 10% [2]. Recent research, however, has shown that immunoparalysis/immunosuppression frequently occurs in patients in the middle and late stages of severe sepsis, with a mortality rate of 70% [3, 4]. This indicates that immunoparalysis may be the main reason for the failure to significantly reduce sepsis mortality clinically by simply suppressing inflammation. Research on sepsis has focused on preventing or reversing sepsis-induced immunoparalysis [5].

Immune cells in the middle and late stages of sepsis often exhibit downregulation of surface co-stimulatory molecules, upregulation of immunosuppressive functions, increased apoptosis and functional depletion, leading to systemic immunoparalysis, ineffective clearance of initial infections, and increased susceptibility to secondary infections. Systemic immunoparalysis is one of the major causes of deadly complications in sepsis patients [6, 7]. Sepsis-induced immunoparalysis is very similar to the pretumor immune microenvironment (pre-TIME) described in cancer [8, 9], which can enable tumor cells to escape surveillance and kill the immune system. Therefore, patients who survive sepsis are more likely to develop cancer [10–12]. Cancer, one of the most important complications of sepsis, accounts

for 25% of mortality after hospital discharge. However, the mechanism underlying this phenomenon is poorly understood, which hinders the development of safe and effective immunotherapies to reverse immunoparalysis and inhibit cancer progression.

CD8⁺T cells have a powerful antitumor immune response [13]. A group reported that sepsis-induced immunoparalysis can be defined by the loss of the antitumor ability of CD8⁺T cells [14]. Myeloid-derived suppressor cells (MDSCs) are functional myeloid cells with immunosuppressive properties [15]. MDSCs can inhibit the proliferation of CD8⁺T cells and the production of IFN- γ , thereby suppressing their antitumor ability [16]. An increase in the number of MDSCs was detected in immunoparalyzed patients after severe sepsis [17], which could be crucial for CD8⁺T-cell exhaustion. Cytokines, among which interleukin (IL)-6 is a pro-cancer cytokine, are currently acknowledged as pivotal mediators of inflammation and cancer [18]. There is a significant correlation between the serum level of IL-6 in sepsis patients at 48 h and their tumor incidence rate [12]. Notably, IL-6 is also the main factor that activates MDSCs and promotes MDSCs proliferation [19].

Recent research has revealed that innate immune cells such as macrophages can be reprogrammed by initial stimuli with immune memory functions, which is called “trained immunity” [20]. Trained immunity can reduce the severity of sepsis and alleviate immunoparalysis in mice [21, 22]. This suggests that trained immunity may be a potential therapeutic strategy to alleviate sepsis-complicated cancer. Oroxylin

A (OA) is an active ingredient extracted from *Scutellaria baicalensis*. OA has a variety of biological effects, such as anti-inflammatory and anti-infection effects [23]. Our previous study revealed that OA can induce macrophages trained immunity and alleviate sepsis in mice [24]. The experimental results showed that trained immunity could lower the serum IL-6 concentration in septic mice. Given the above findings, it is reasonable to hypothesize that OA-induced trained immunity might inhibit sepsis-induced MDSCs proliferation and enhance the antitumor function of CD8⁺T cells, reversing the pre-TIME induced by severe sepsis.

To address the hypothesis that sepsis-induced immunoparalysis promotes tumor progression via dysregulation of immunosuppressive pathways, this study aims to systematically investigate the underlying mechanisms using two sepsis-melanoma mouse models. Specifically, we determined whether elevated serum IL-6 levels, an altered G-MDSCs/M-MDSCs ratio, and PD-L1 upregulation in G-MDSCs during the immunoparalysis phase collectively drive CD8⁺T cell exhaustion and functional impairment, thereby facilitating melanoma growth. Furthermore, we explored the therapeutic potential of Oroxylin A (OA) to counteract immunoparalysis by inducing trained immunity in macrophages, with a focus on its ability to modulate key targets such as MIF and IL-6. This work aims to clarify the mechanistic link between sepsis-associated immunoparalysis and cancer progression while evaluating trained immunity as a novel strategy to restore anti-cancer immunity in sepsis survivors.

2 Methods

2.1 Cell line

B16F10 cells were cultured in complete medium (1640, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (100 µg/mL; Gibco BRL, USA). The incubation took place at 37 °C with 5% CO₂. All experiments were performed with mycoplasma-free cells.

2.2 Mice

We obtained C57BL/6 male mice (20–25 g, 6–8 weeks old) from Nanjing Cavans Biotechnology Co, Ltd. (Nanjing, China). The mice were housed in SPF conditions (18–25 °C, 40–70% humidity) and fed a regular schedule. All experimental protocols were approved by the Animal Protection and Ethics Committee of Nanjing University (IACUC-D2202077), and mice were euthanized at the experiment's conclusion.

2.3 Reagents

Professor Qinglong Guo from China Pharmaceutical University (Nanjing, China) generously provided OA, which was stored at -80 °C in dimethyl sulfoxide (DMSO). In vitro, OA was dissolved in DMSO/PBS, maintaining DMSO concentration below 0.1%. OA, purified from *Scutellaria baicalensis* root (purity ≥ 99%) [25], was used in all experiments. For in vivo use, OA was configured with 0.5% Carboxymethyl cellulose-Na (CMC-Na) (Sigma, Shanghai, China). LPS (Sigma, Shanghai, China), stored at -20 °C, was diluted in PBS to the required concentration prior to use. ISO-1 (MCE, China) stored at -20 °C, was diluted in CMC-Na to the required concentration prior to use.

2.4 Experimental sepsis

At the conclusion of trained immunity, mice received 5 mg/kg LPS intraperitoneally to induce endotoxemia. Polymicrobial sepsis was modeled through cecal ligation and puncture (CLP) surgery. Mice, anesthetized with isoflurane, underwent a 1 cm incision below the diaphragm, where the cecum was ligated and punctured. The sham group underwent the same procedure without ligation and puncture.

2.5 Melanoma model

B16F10 cells in logarithmic growth were harvested. After making a cell suspension, 5×10^5 B16F10 cells in 100 µL PBS were subcutaneously injected on the right side of mice to establish a subcutaneous melanoma model on day 2 or day 4 post-sepsis model construction. The experiment followed aseptic procedures. Once tumors were visible, mouse weight, tumor long (L), and short (W) diameters were measured using a weight apparatus and vernier calipers. Tumor volume (V) was calculated as $V = (LW^2)/2$.

2.6 Trained immunity in vivo

Mice received intraperitoneal injections of PBS, OA (40 mg/kg) or BG (35 mg/kg) twice weekly on Day -7 and Day -3 before LPS (5 mg/kg) injection or the CLP procedure, following established protocols [24]. This regimen facilitates the development of an immune memory response, where innate immune cells, particularly monocytes and macrophages, undergo functional reprogramming. Previous researchers have also used this method to induce in vivo training immunity in mice [21, 22].

2.7 Macrophage clearance model

Mice received intraperitoneal injections of 100 μ L Clophosome[®]-A (CLL) (FormuMAX) to deplete macrophages, and control group mice received 100 μ L con-CLL for confirmation of macrophage clearance efficiency.

2.8 Analysis of infiltrating immunocytes by flow cytometry

After counting single cell suspensions from various tissues [26], $1-2 \times 10^6$ cells were added to each tube. eBioscience[™] Cell Stimulation Cocktail (plus protein transport inhibitors) (Invitrogen, USA) was used to detect the expression of IFN- γ in CD8⁺T cells. Apply surface flow cytometry antibodies as required, following dosage instructions. Incubate for 30 min at 4°C in the dark, then wash with PBS. For staining intracellular proteins, treat cells with a membrane-breaking solution for 1 h, followed by washing and staining with corresponding flow cytometry antibodies under the same conditions. The samples were analysed by flow cytometry (BECKMAN, Cytoflex, Beckman Coulter Cytoflex S). Flow cytometric data were analyzed using FlowJo software (Treestar, Ashland, OR, USA). Antibodies used for flow cytometry are listed in Supplementary Table 1.

2.9 Immunohistochemistry(IHC)

Tissue sections (2 μ m) were incubated overnight at 4 °C with primary antibodies against Ki67 or macrophage migration inhibitory factor (MIF). Antigen signals were visualized using DAB after exposure to HRP-conjugated secondary antibodies.

2.10 Immunofluorescence

Tumor tissues were fixed in 4% PFA immediately after ex vivo and sent to FreeThinking biotechnology CO.,TLTD for paraffin embedding and sectioning. After the sections were deparaffinised and recovered, the primary antibody was added and incubated at 4 °C overnight. After incubation, the slices were washed three times with PBS, fluorescent secondary antibody was added, and incubated at room temperature for 2 h. After incubation, the slices were washed and sealed with anti-fluorescence quencher. Scanning was performed with 3DHISTECH (Pannoramic MIDI II), and SlideViewer software was used for observation.

2.11 RNA extraction and qRT-PCR

Total RNA was extracted using Trizol Reagent (Vazyme, Nanjing, China), followed by cDNA synthesis with the

Fig. 1 Melanoma progression is increased after sepsis. (A) Schematic diagram of the construction of LPS-melanoma mouse model ($n=6$). (B) Representative diagrams of melanoma tissues obtained from different groups. (C) Comparison of melanoma weights. (D) Comparison of melanoma growth curves. (E) Curve of body weight changes in mice. (F) Ki67 protein expression detected by immunohistochemistry and quantitative analysis. (G) The expression level of IL-6 in serum detected by ELISA. (H) Schematic diagram of the construction of CLP-melanoma mouse model ($n=5$). (I) Representative diagrams of melanoma tissues obtained from different groups. (J) Comparison of melanoma weights. (K) Melanoma growth curves. (L) Body weight change curves of the mice. (M) Ki67 protein expression detected by immunohistochemistry and quantitative analysis. (N) The expression level of IL-6 in serum detected by ELISA (left 2Day groups, right 4Day groups). Bar = 0.1 mm. The measurement data are presented as the means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

HiScript[®] II Q RT SuperMix kit (Vazyme Nanjing, China). qRT-QPCR was conducted using SYBR Green dye (Invitrogen) on a StepOne sequence detection system (Applied Biosystems, Waltham, MA, USA). The $2^{-\Delta\Delta CT}$ algorithm, with β -actin as the internal reference, determined the relative gene abundances. qRT-PCR primer sequences are provided in Supplementary Table 2.

2.12 Enzyme-linked immunosorbent assay (ELISA)

The instructions were followed while using ELISA kits (IL-6, TNF- α : BioLegend; IL-1 β : R&D; MIF: MEIMIAN).

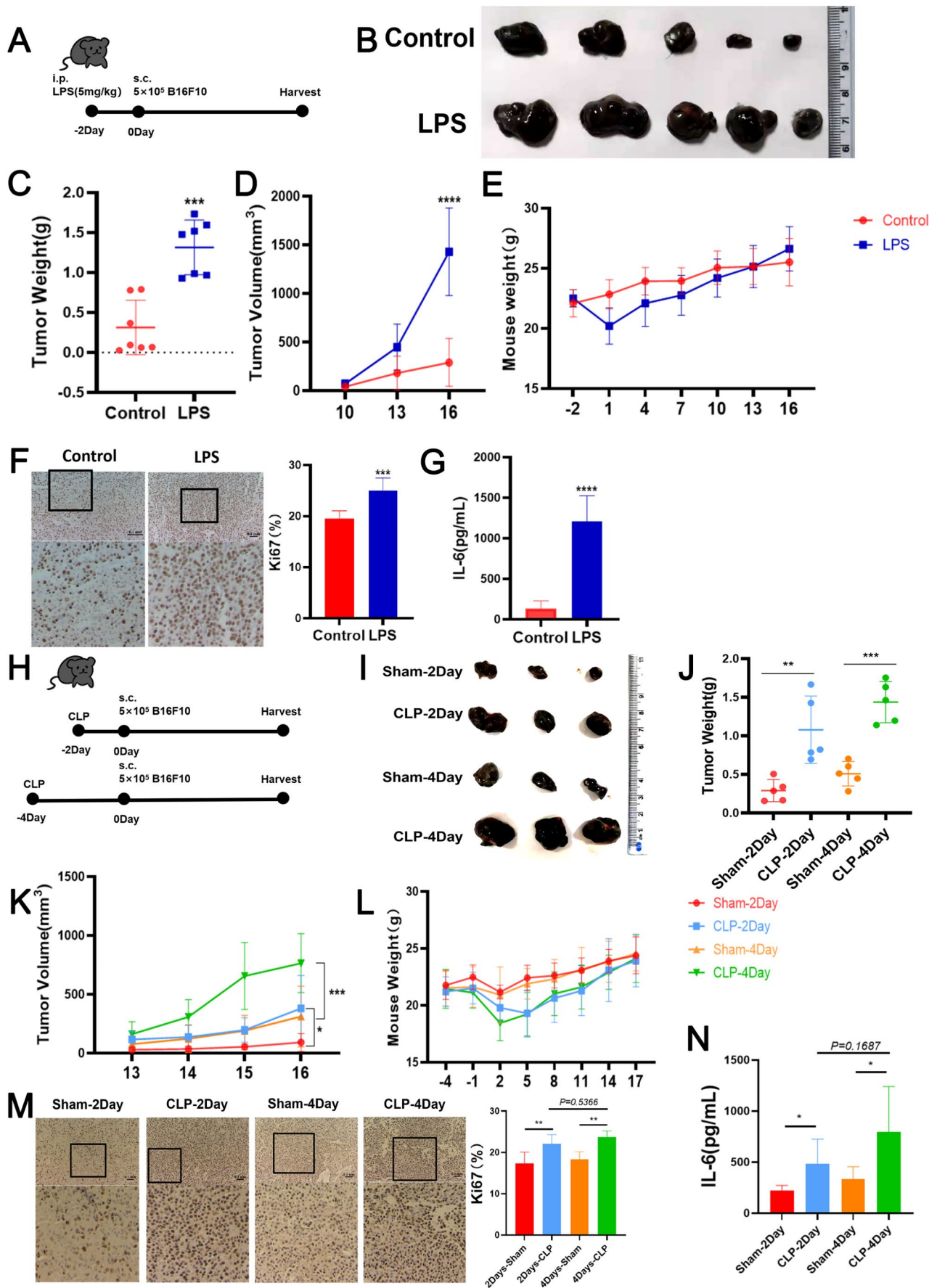
2.13 Statistical analysis

All experiments were randomized and blinded, repeated at least three independent times. Graphs depict means \pm SEM. The Shapiro–Wilk test was used to confirm whether the data were normally distributed. One-way ANOVA was used to analyses the data among more than two groups, and Student's t tests were used to compare two groups. * $P < 0.05$ indicates statistical significance. Statistical analysis was performed using GraphPad Prism 5 Demo.

3 Results

3.1 Sepsis can enhance melanoma progression

For melanoma growth to occur primarily during the period of immunoparalysis, we s.c. the hind flank with 5×10^5 B16F10 cells 2 days after LPS injection (Fig. 1A) [14]. The results showed that sepsis significantly promoted the progression of melanoma in mice (LPS group), increasing tumor weight and volume (Fig. 1B–D). Although there was no significant difference in body weight between control and LPS group mice at the end of the experiment, we observed a faster rate of weight gain in the LPS group than



in the control group, which may be related to accelerated melanoma growth (Fig. 1E). IHC analysis further confirmed that cancer cells proliferation (Ki67 positive) in the LPS group was significantly greater than that in the control group (Fig. 1F). Moreover, the serum level of IL-6 was significantly elevated in the LPS group (Fig. 1G), while the serum levels of IL-1 β and TNF- α showed a decreasing trend or a nonsignificant difference (Supplementary Fig. 1A-B).

To confirm whether this phenomenon is also prevalent in the polymicrobial sepsis model, we s.c. 5×10^5 B16F10 cells on 2 or 4 days after CLP/sham surgery (Fig. 1H). CLP promoted melanoma progression and increased melanoma weight (Fig. 1I-K). However, no significant difference in body weight was found between the CLP group and the sham group (Fig. 1L). The serum level of IL-6 was also markedly increased in the CLP group (Fig. 1N), while the level of IL-1 β showed a decreasing trend or a nonsignificant difference (Supplementary Fig. 1C). Melanoma proliferation in the CLP group was significantly greater than that in the Sham group (Fig. 1M). These data showed that sepsis could induce the progression of melanoma, which was related to IL-6.

3.2 Melanoma progression is associated with sepsis-induced immunoparalysis

To further explore this phenomenon, we examined the ratio of MDSCs and CD8⁺T cells in melanoma and spleen. As expected, the proportions of MDSCs in the melanoma (Fig. 2A, D) and spleen (Fig. 2C, E) were significantly increased in both sepsis models. We observed a marked decrease in the percentage of CD8⁺T cells in melanoma, and the IFN- γ production capacity was significantly suppressed in septic immunoparalysed stage (Fig. 2B, F). An increase in the proportion of MDSCs, a decrease in the proportion of CD8⁺IFN- γ ⁺T cells and an increase in serum IL-6 levels were associated with accelerated melanoma progression. Additionally, we observed reduced levels of anti-cancer cytokines in the sepsis group at the gene level, coupled with an increase in IL-6, reinforcing our experimental findings (Supplementary Fig. 2). Thus, the data collectively showed that sepsis induced immunoparalysis by regulating IL-6, MDSCs and CD8⁺IFN- γ ⁺ T cells, which in turn promoted melanoma progression.

3.3 Sepsis-induced melanoma progression is alleviated by OA-induced trained immunity

Our group previously reported that OA could induce trained immunity and alleviate sepsis in mice [24]. To further investigate the key characteristics of OA-induced trained immunity, we performed CUT&Tag sequencing for H3K27ac

to examine epigenetic changes in macrophages [27–29]. Our results demonstrated that Pearson's correlation analysis among different samples showed high reproducibility between biological replicates, ensuring the reliability of the CUT&Tag data. Additionally, the expression pattern in the OA group was more similar to that in the BG group than in the control group (Fig. 3A). Compared to the control group, H3K27ac enrichment was markedly increased in both the OA and BG groups, indicating enhanced chromatin accessibility in response to trained immunity stimulation (Fig. 3B). Heatmap analysis of H3K27ac peaks around transcription start sites (TSSs) (-5 to +5 kb) revealed a global increase in acetylation levels in macrophages treated with OA or BG, further confirming chromatin remodeling induced by trained immunity (Fig. 3C).

Genomic feature distribution analysis indicated that H3K27ac peaks were predominantly enriched in promoter regions, with the OA group exhibiting an increased proportion of peaks in regulatory elements, underscoring its role in transcriptional activation (Fig. 3D). Epigenetic activation of inflammatory genes such as *Il1b*, *Tnf*, and *Il18* is one of the markers of trained immunity [27]. IGV genome browser tracks demonstrated prominent H3K27ac enrichment at the promoters of these key inflammatory genes in the OA group, suggesting that OA enhanced the chromatin accessibility of these loci, thereby promoting their transcriptional activation (Fig. 3E).

Functional enrichment analysis (KEGG and GO) of unique peak-associated genes in the OA group revealed significant enrichment in immune activation and inflammatory response pathways, including the TNF signaling pathway, MAPK signaling pathway, and FoxO signaling pathway, suggesting potential molecular mechanisms through which OA-induced trained immunity (Fig. 3F-G). In summary, these findings indicated that OA-induced trained immunity in macrophages by promoting H3K27ac-mediated epigenetic reprogramming, thereby enhancing the accessibility of key immune-related genes.

We established a mouse model of sepsis with OA-induced trained immunity. Then, 5×10^5 B16F10 cells were s.c. into the hind flank after LPS injection on 2 day (OA+LPS group) (Fig. 4A). β -glucan, a trained immunity-positive drug, was used as a positive control (BG+LPS group) [30]. As expected, OA/BG-induced trained immunity alleviated the accelerated progression of melanoma and reduced melanoma weight and size (Fig. 4B-D). Moreover, OA-induced trained immunity significantly alleviated sepsis-induced weight loss in mice, demonstrating the success of our trained immunity model (Fig. 4E). Compared to that in the LPS group, the serum level of IL-6 in the OA+LPS group and BG+LPS group was markedly lower (Fig. 4F), which was consistent with the decreased melanoma Ki67 in

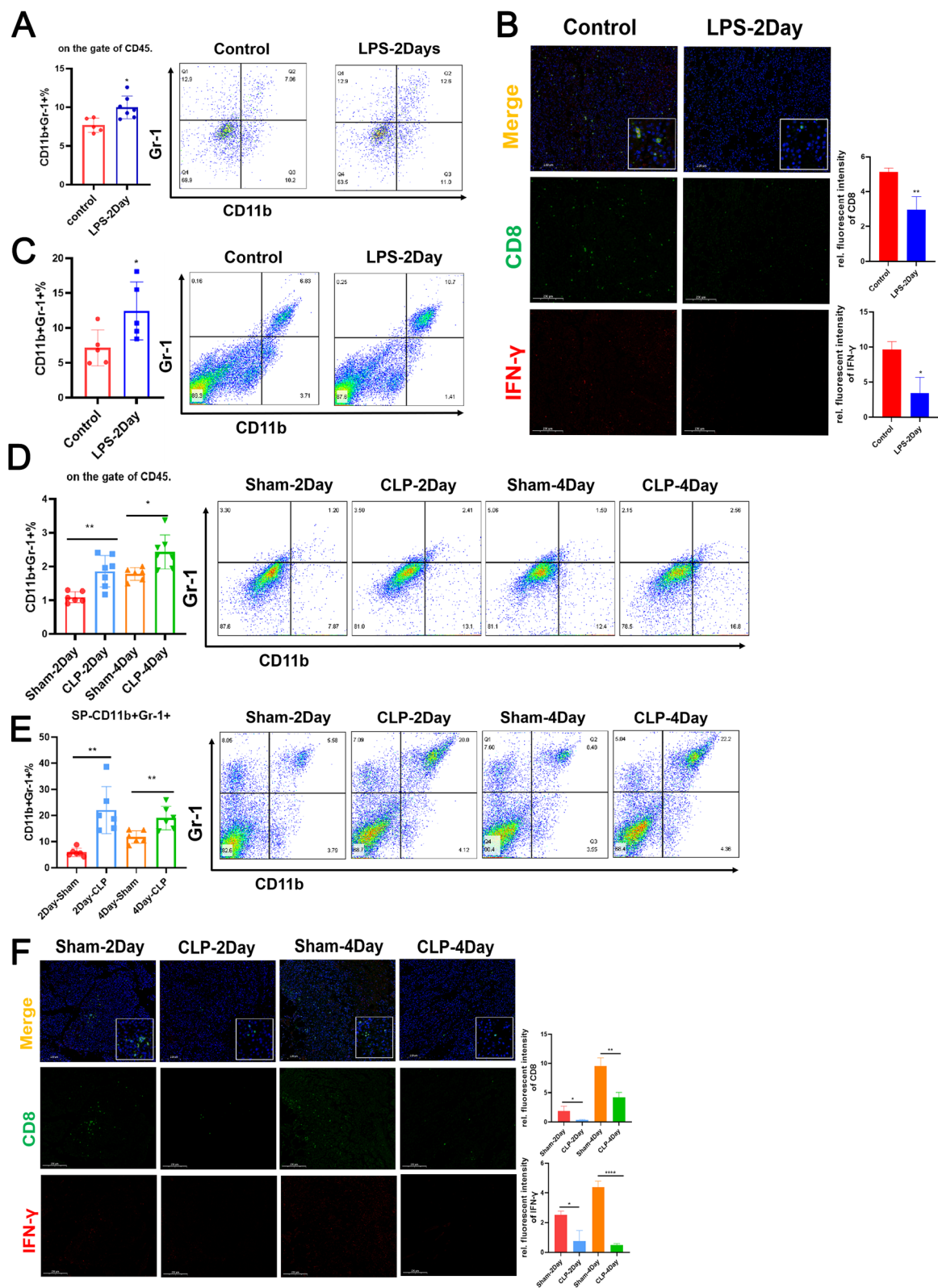


Fig. 2 Melanoma progression is associated with the immunosuppressive function of MDSCs. The ratio of MDSCs in melanoma (A) or spleen (C) was detected FCM in LPS-melanoma group. (B) Immunofluorescence was used to detect the expression of CD8 and IFN- γ in tumor tissues. The ratio of MDSCs in melanoma (D) or spleen (E)

was detected FCM in CLP-melanoma group. (F) Immunofluorescence was used to detect the expression of CD8 and IFN- γ in tumor tissues. The measurement data are presented as the means \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

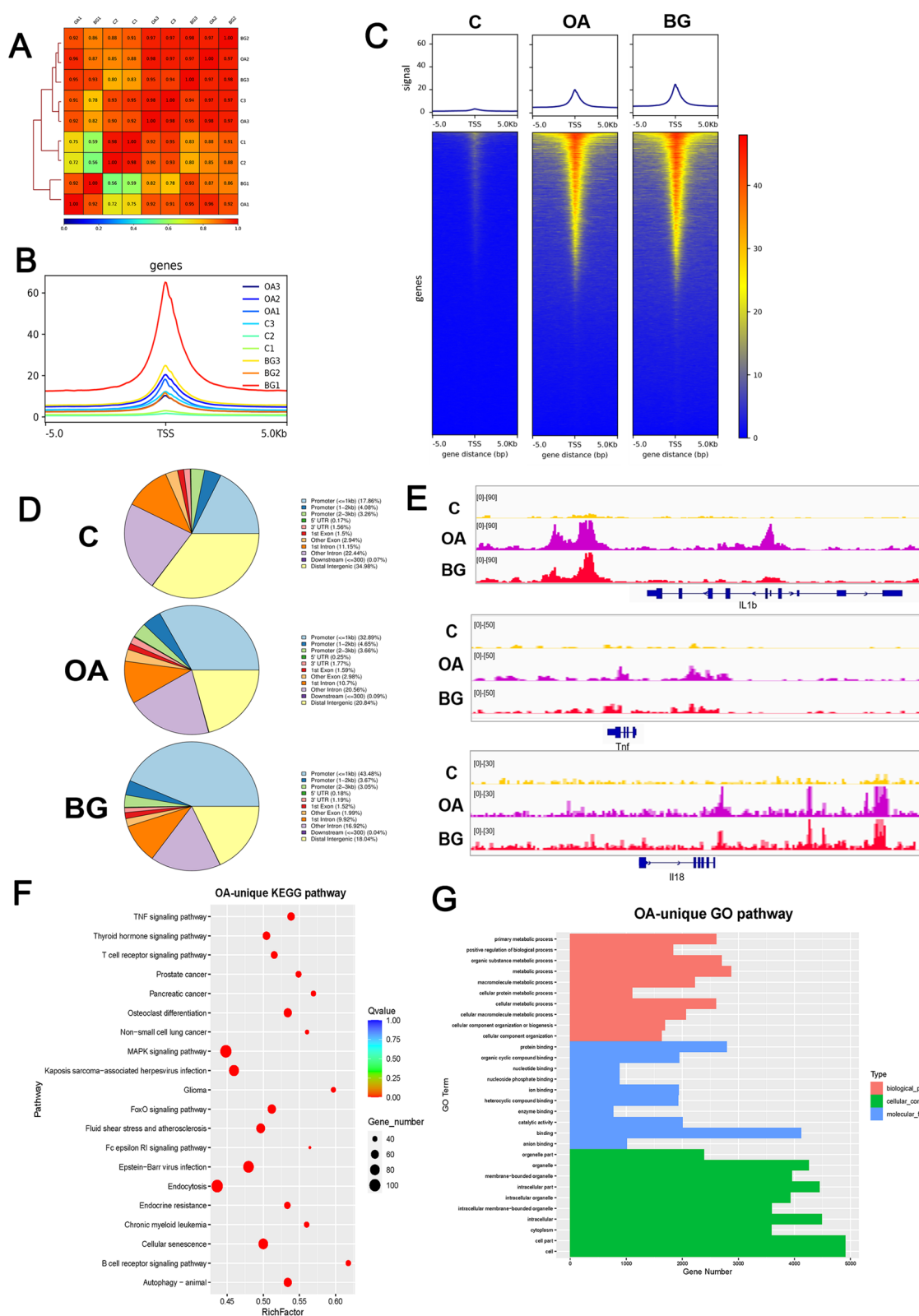


Fig. 3 OA-induced trained immunity is epigenetically mediated by enhancing H3K27ac in macrophages. **(A)** Pearson's coefficient across samples. **(B)** Peak coverage plots of H3K27ac upon control, OA or BG group in J774A.1. **(C)** Heatmap of H3K27ac peaks in the TSSs. Rows are genomic regions from -5 to +5 kb around the center of the peaks. **(D)** The proportion of peaks assigned to genomic features in differ-

ent group. **(E)** IGV genome browser tracks representing the H3K27ac CUT&Tag peaks of IL1b, Tnf and Il18 in J774A.1 ($n=3$). **(F)** The KEGG enrichment analysis of the unique peak-related genes in OA group. **(G)** The GO enrichment analysis of the unique peak-related genes in OA group

the OA+LPS group and BG+LPS group (Fig. 4G). Complying with MDSCs naming and research reports [31, 32], we replaced the flow cytometry gating strategy for MDSCs to typify MDSCs and selected PD-L1 as additional marker to define MDSCs inhibition of CD8⁺T-cell function (Supplementary Fig. 3A). PD-L1 inhibits the killing function of CD8⁺ T cells and depletes CD8⁺ T cells, promoting tumor growth and immune escape [33]. The flow cytometry gating sequence for CD8⁺IFN- γ ⁺ T cells is shown in Supplementary Fig. 3B.

In our study, G-MDSCs/M-MDSCs were significantly increased in cancer in the immunoparalysis stage, indicating that the proliferation of G-MDSCs in this stage was associated with accelerated cancer growth in the immunoparalysis stage (Fig. 4H). PD-L1 expression was also significantly upregulated in G-MDSCs (Figure re 4 I), corresponding to impaired function of CD8⁺ T cells (Fig. 4J). G-MDSCs in the spleen showed the same trend (Fig. 4K-L), and G-MDSCs also inhibited the function of CD8⁺ T cells (Fig. 4M). In contrast, OA-induced trained immunity significantly reversed this phenomenon (Fig. 4H-M). It has been reported that the proportion of G-MDSCs is increased after CLP surgery, and these cells constitute the main group of MDSCs expressing PD-L1, which is similar to our experimental results [34]. The results of immunofluorescence colocalization showed that G-MDSCs (Ly6G⁺) was present in both tumor tissue and partial vasculature of the tumor (CD31⁺), suggesting G-MDSCs may undergo migration from the peripheral blood into the tumor tissue (Supplementary Fig. 3C).

It has been reported that trained immunity can directly inhibit tumor growth [35]. To exclude a direct inhibitory effect of trained immunity on tumor growth, we subcutaneously s.c. 5×10^5 B16F10 cells in trained immunity mice (without sepsis). There were no significant changes in tumor size or weight, tumor proliferative capacity or serum levels of IL-6 in the OA or BG group compared to those in the control group (Fig. 4B-G). There were also no significant differences between G-MDSCs/M-MDSCs (Fig. 4H), the immunosuppressive capacity of G-MDSCs (Fig. 4I) or the function of CD8⁺ T cells (Fig. 4J). The slower growth of tumors may be due to mice under the non-immunoparalysis phase, and differences between the groups had not yet been apparent at the time of sampling. Nevertheless, our results suggest the trained immunity might reverse sepsis-induced immune paralysis to inhibit tumor progression but not be a direct tumor-suppressive effect of trained immunity.

OA-induced trained immunity creates an immune environment that inhibits melanoma progression. Collectively, these findings showed that OA-induced trained immunity can inhibit the distribution of G-MDSCs, inhibit the expression of PD-L1 and enhance the function of CD8⁺T cells (the

ability to secrete IFN- γ), reversing sepsis-induced accelerated melanoma progression.

3.4 OA-induced trained immunity reduces sepsis-induced immunoparalysis by targeting MIF

Following the observation that OA-induced trained immunity could reverse sepsis-induced immunoparalysis, we examined the functional target of this phenomenon. The results revealed the presence of active binding sites between OA and MIF (Fig. 5A). MIF is an important therapeutic target in sepsis, and higher levels of MIF in the peripheral blood are associated with a worse prognosis [36]. Furthermore, MIF is closely associated with the growth and metastasis of many tumors, and inhibiting MIF can suppress cancer progression [37].

In the LPS group, the mRNA level of MIF in the spleen was greater than that in the control group, and OA-induced trained immunity suppressed this increase (Fig. 5B). The difference in the protein level of MIF in melanoma samples was the same between the IHC (Fig. 5C) and WB (Supplementary Fig. 4A) results, and also showed that the protein of MIF was greater than that in the control group, and OA-induced trained immunity suppressed this increase. These data further confirmed that OA-induced trained immunity could inhibit sepsis-induced MIF expression. Furthermore, database analysis revealed that MIF was expressed at high levels in melanoma patients (Fig. 5D), and higher MIF expression usually indicated poorer survival (Fig. 5E). In addition, MIF was highly expressed in most cancers (Supplementary Fig. 4B).

There was no difference between the OA+MIF group and the OA+LPS group (Supplementary Fig. 4C-G). However, compared with that in the LPS group, the flow cytometry results showed that although the G-MDSCs/M-MDSCs was significantly decreased in the OA+MIF group, the ratio of PD-L1⁺G-MDSCs in the OA+MIF group was not significantly different from that in the LPS group (Supplementary Fig. 4I). The proportion of CD8⁺IFN- γ ⁺T in tumors (Supplementary Fig. 4J) and spleens (Supplementary Fig. 4M) were also not significantly different from those in the LPS group. This suggests to us that OA may inhibit G-MDSCs production by directly binding to MIF but only partially alleviates the immunosuppressive environment.

3.5 OA influences the post-sepsis immunosuppressive environment through macrophages

Accelerated melanoma progression after sepsis is closely related to the sepsis-induced state of immunoparalysis, which can be reversed by trained immunity. Our

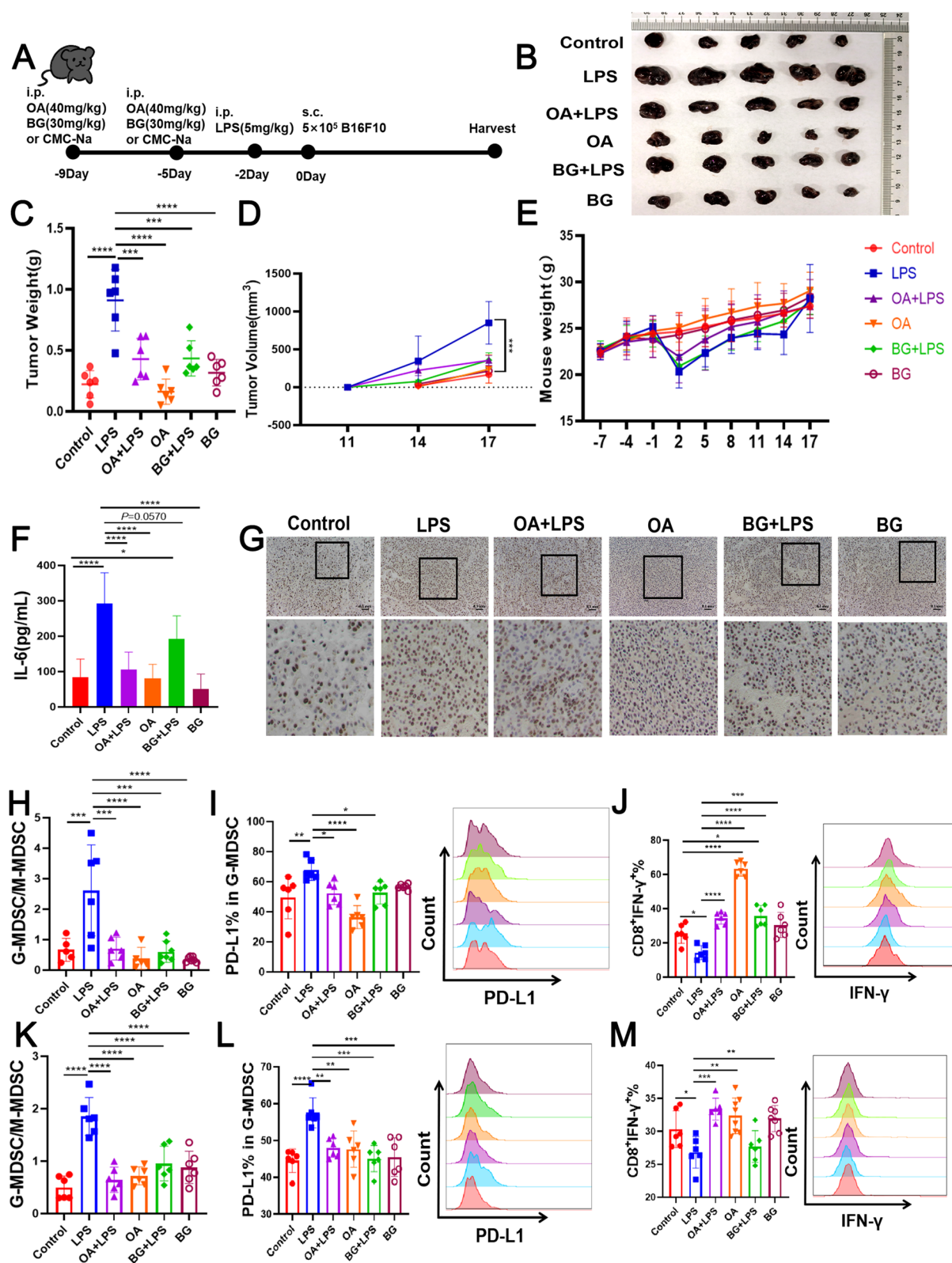


Fig. 4 Sepsis-induced melanoma progression is alleviated by OA-induced trained immunity. (A) Schematic diagram of the construction of LPS-melanoma mouse model ($n=6$). (B) Representative diagrams of melanoma tissues, (C) comparison of melanoma weights and (D) melanoma growth curves. (E) Curve of body weight changes in mice. (F) The expression level of IL-6 in serum detected by ELISA. (G) Ki67 protein expression detected by immunohistochemistry. The ratio of (H) G-MDSC/M-MDSC, (I) PD-L1⁺G-MDSC or (J) CD8⁺IFN- γ ⁺T cells in tumor tissue were detected FCM. The ratio of (K) G-MDSC/M-MDSC, (L) PD-L1⁺G-MDSC or (M) CD8⁺IFN- γ ⁺T cells in spleen were detected FCM. Bar = 0.1 mm. The measurement data are presented as the means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

experimental results showed that trained immunity reduced the expression of IL-6 and MIF in serum for at least 72 h after LPS injection (Fig. 6A-B) and was temporally positively correlated with the downregulation of MDSCs and the upregulation of CD8⁺ T cells (Fig. 6C-D). We analyzed the degree of lymphopenia. There was no significant difference in the number of lymphocytes between the sepsis group (LPS-1Day group) and the trained immunity-related sepsis group (OA-1Day group) on the first day after sepsis induction. From the second day, the number of lymphocytes in the OA-2Day group was significantly greater than that in the LPS-2Day group (Fig. 6E), suggesting the recovery of immune function in the mice. This result suggested that OA-induced trained immunity could reverse sepsis-induced immunoparalysis, which might be the key to its ability to inhibit accelerated melanoma progression.

Macrophages are an important source of MIF. Moreover, OA induces trained immunity targeting of innate immune cells. OA-induced trained immunity inhibited the LPS-induced increase in MIF expression in macrophages in vitro (Fig. 6F). Therefore, we hypothesized that OA might target MIF in macrophages to regulate the growth of advanced melanoma in sepsis. We established a sepsis model with OA-induced trained immunity or the removal of macrophages by Clodronate Liposomes (CLL) (Fig. 7A). The results of ELISA experiments showed that the serum levels of MIF and IL-6 were still increased after sepsis, but trained immunity or the removal of macrophages reversed this phenomenon (Fig. 7B-C). The flow cytometry results showed that the proportions of MDSCs in the spleen and bone marrow were significantly increased and that the proportion of CD8⁺T cells was significantly decreased after sepsis. However, trained immunity or macrophages clearance inhibited the proliferation of MDSCs and increased the proportion of CD8⁺ T cells (Fig. 7D-G). There were significantly fewer macrophages in the spleen and bone marrow in the CLL sepsis group than in the sepsis group (Fig. 7H-I). These results suggested that macrophages play an important role in sepsis-induced immunoparalysis, which might be related to MIF secreted by macrophages during sepsis.

3.6 OA affects the immune microenvironment during melanoma progression by targeting the MIF-IL-6 axis

To formally prove the importance of MIF in sepsis-induced melanoma progression, we treated mice with the MIF inhibitor ISO-1 to establish a sepsis-melanoma model (Fig. 8A). We observed that inhibition of MIF markedly reversed sepsis-induced melanoma progression (Fig. 8B-D), reduced melanoma weight (Fig. 8C), and decreased serum levels of IL-6 (Fig. 8F). However, ISO-1 treatment did not alleviate sepsis-induced weight loss in mice, which further demonstrated the superiority of trained immunity in alleviating sepsis in mice (Fig. 8E).

Furthermore, we performed flow cytometry to assess the distribution of MDSCs and CD8⁺ T cells in melanoma and the spleen. The results showed that inhibition of MIF decreased the proportion of G-MDSCs/M-MDSCs (Fig. 8G, J), inhibited the expression of PD-L1 in G-MDSCs (Fig. 8H, K), and increased the expression of IFN- γ in CD8⁺T cells (Fig. 8I, L) in the melanoma and spleen. These results suggested that MIF originated from macrophages and regulated the secretion of IL-6, which affects G-MDSCs and CD8⁺IFN- γ ⁺T cells. Ultimately, a high level of MIF can induce immunoparalysis that promotes the progression of melanoma. OA-induced trained immunity reversed the accelerated progression of sepsis-induced melanoma by targeting the MIF-IL-6 axis.

4 Discussion

Sepsis involves complex pathophysiologic mechanisms [1]. Previously, it was widely considered that an excessive inflammatory response is closely related to organ damage in sepsis. However, in recent years, an increasing number of studies and clinical data have shown that post-sepsis immunoparalysis/immunosuppression can be a major cause of high mortality and poor prognosis in sepsis patients [38, 39]. Due to the important role of the healthy immune system in anti-cancer surveillance, sepsis immunoparalysis is often accompanied by a high incidence of cancer. Some recent clinical data have shown that a high incidence of myeloid malignancies occurs in patients with a history of sepsis prior to the diagnosis of malignancy [40, 41]. There is also an increased risk of epithelial cancer in patients with pre-existing sepsis [10]. In our study, in both the LPS-induced endotoxemia and CLP surgery-induced poly-bacteremia models, sepsis promoted melanoma development, increasing tumor size, weight, and proliferation. The number of MDSCs has been reported to increase persistently during sepsis, inducing a state of immunoparalysis [34]. It has been shown that

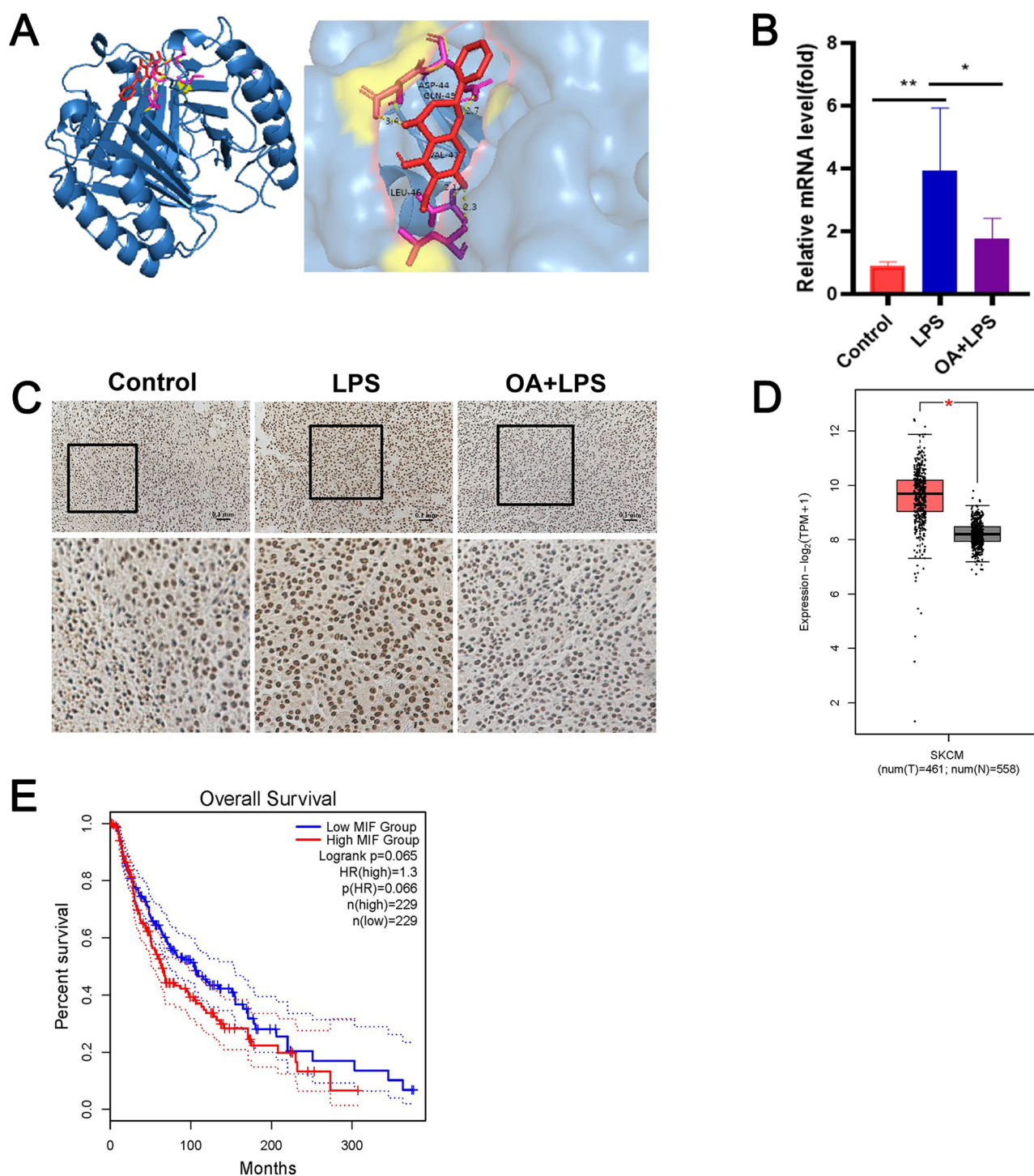


Fig. 5 Changes in MIF expression and database analysis in OA-induced trained immunity in the sepsis-melanoma model. **(A)** Molecular docking of OA and MIF. **(B)** mRNA expression of MIF in spleen. **(C)** MIF protein expression detected by immunohistochemistry. Bioinformatics analysis of MIF expression in **(D)** melanoma-like clinical

samples and paracancerous tissues in databases, and **(E)** analysis of MIF expression in melanoma in relation to survival. Bar = 0.1 mm. The measurement data are presented as the means \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

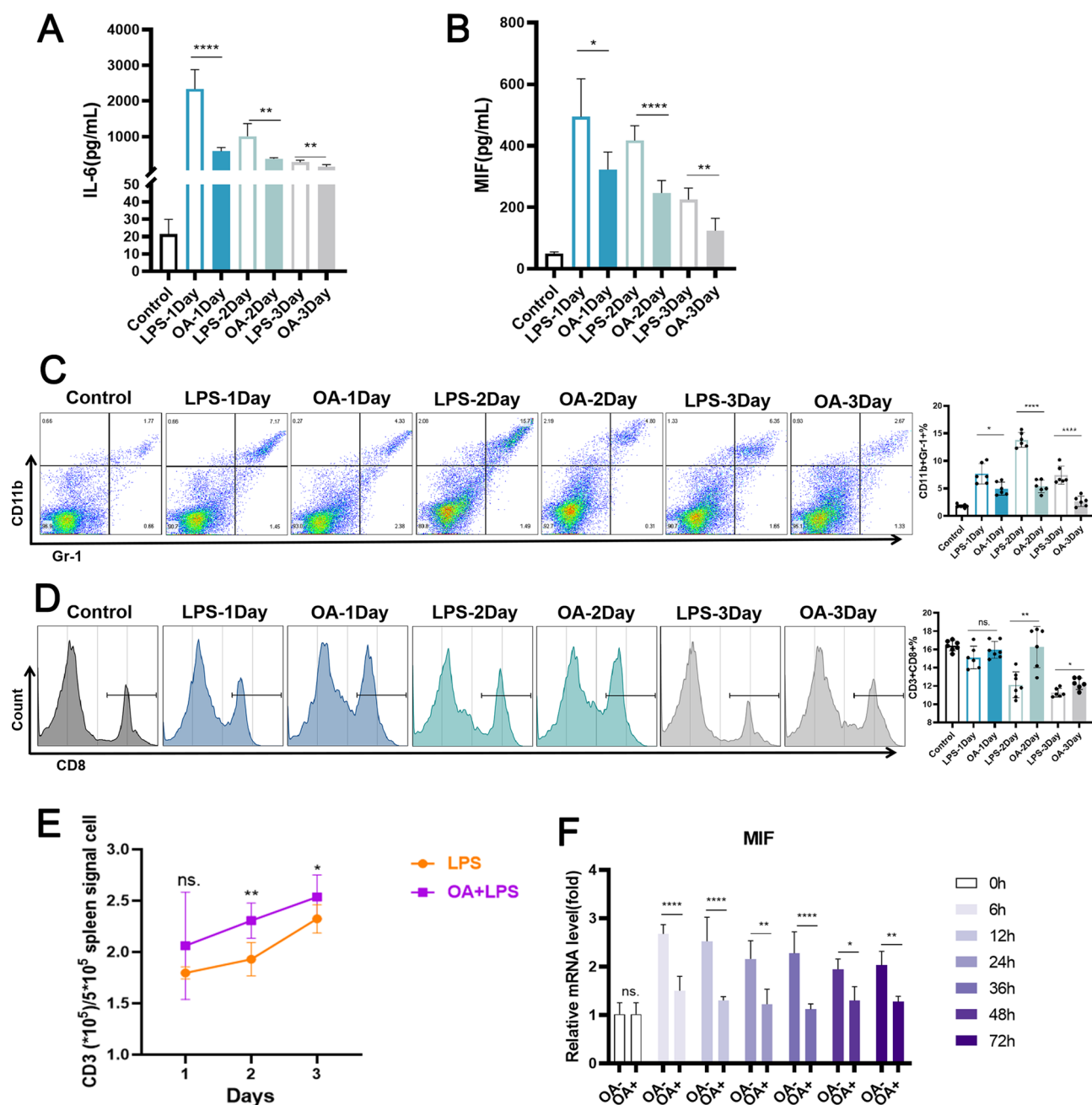


Fig. 6 Distribution of MDSCs and CD8⁺T cells in relation to time after sepsis. The expression level of (A) IL-6 and (B) MIF in serum detected by ELISA. (C) FCM results of the distribution of MDSCs in mouse spleen. (D) FCM results of the distribution of CD8⁺T cells in mouse

spleen. (E) The degree of lymphopenia in the septic mice spleen. (F) Temporal expression of MIF at the mRNA levels in macrophages in vitro. The measurement data are presented as the means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

the antitumor capacity of CD8⁺T cells is diminished during sepsis-induced immunoparalysis, as shown by reduced tumor infiltration of CD8⁺T cells and reduced cell proliferation and survival [14]. Similarly, the results of our experiments also showed that sepsis induces the proliferation of MDSCs, increases the ratio of G-MDSCs, and decreases the infiltration of CD8⁺IFN- γ ⁺ T cells in melanoma and

the spleen, which is accompanied by accelerated growth of melanoma.

Trained immunity is an emerging means of inducing the reprogramming of innate immune cells (e.g., macrophages) [20]. Trained immunity is thought to result in long-term changes in innate immune cells rather than transient changes induced by a single transcription factor. It is regulated through sustained changes in epigenetic pathways

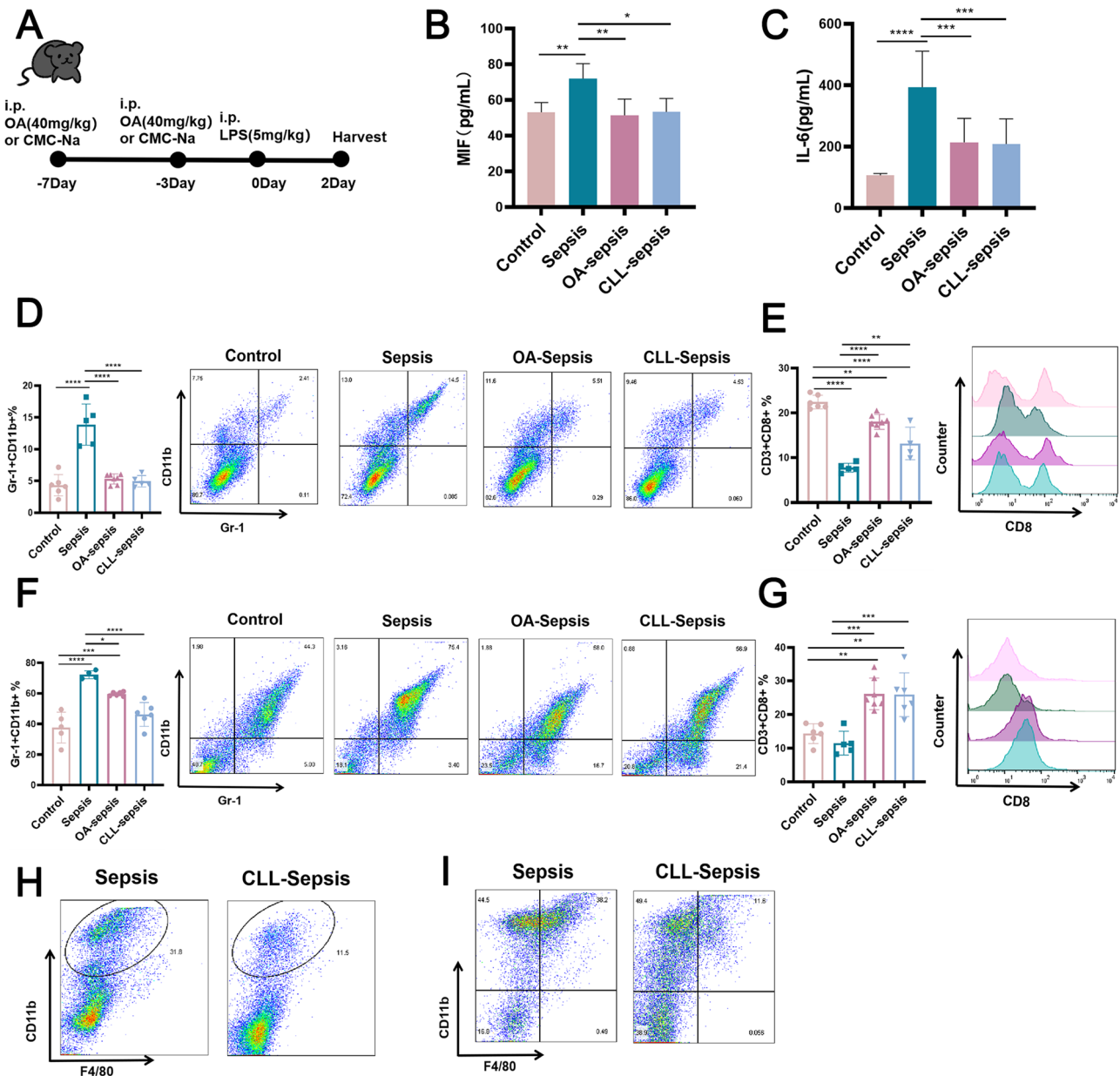


Fig. 7 Immune status in sepsis after trained immunity/clearance of macrophage. **(A)** Schematic diagram of the construction of sepsis model ($n=5-7$). The expression level of **(B)** MIF and **(C)** IL-6 in serum detected by ELISA. The distribution of **(D)** MDSCs or **(E)** CD8⁺T cells in spleen was detected FCM. The distribution of **(F)** MDSCs or

(G) CD8⁺T cells in BMDM was detected FCM. Efficiency of CLL clearance of **(H)** spleen and **(I)** bone marrow macrophages detected by FCM. The measurement data are presented as the means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

and metabolic pathways. When they are re-stimulated with different stimuli, innate immune cells will respond more strongly [42, 43]. β -Glucan is recognized as a positive drug that induces trained immunity [44]. We previously reported that β -glucan-induced macrophage trained immunity could alleviate sepsis and prevent secondary infection [21, 22]. In addition, our group has innovatively discovered that OA can induce trained immunity in macrophages and alleviate sepsis by enhancing macrophage LC3-associated phagocytosis

[24]. This suggested that small molecule compounds can also induce trained immunity, like OA and glucocorticoids [45]. In this study, we further found that OA-induced trained immunity reversed sepsis-induced immunoparalysis, as evidenced by a decrease in the percentage of G-MDSCs and increased tumor infiltration of CD8⁺IFN- γ ⁺ T cells. This could reverse the sepsis-induced tumor progression. We observed that the size and weight of melanoma in mice in

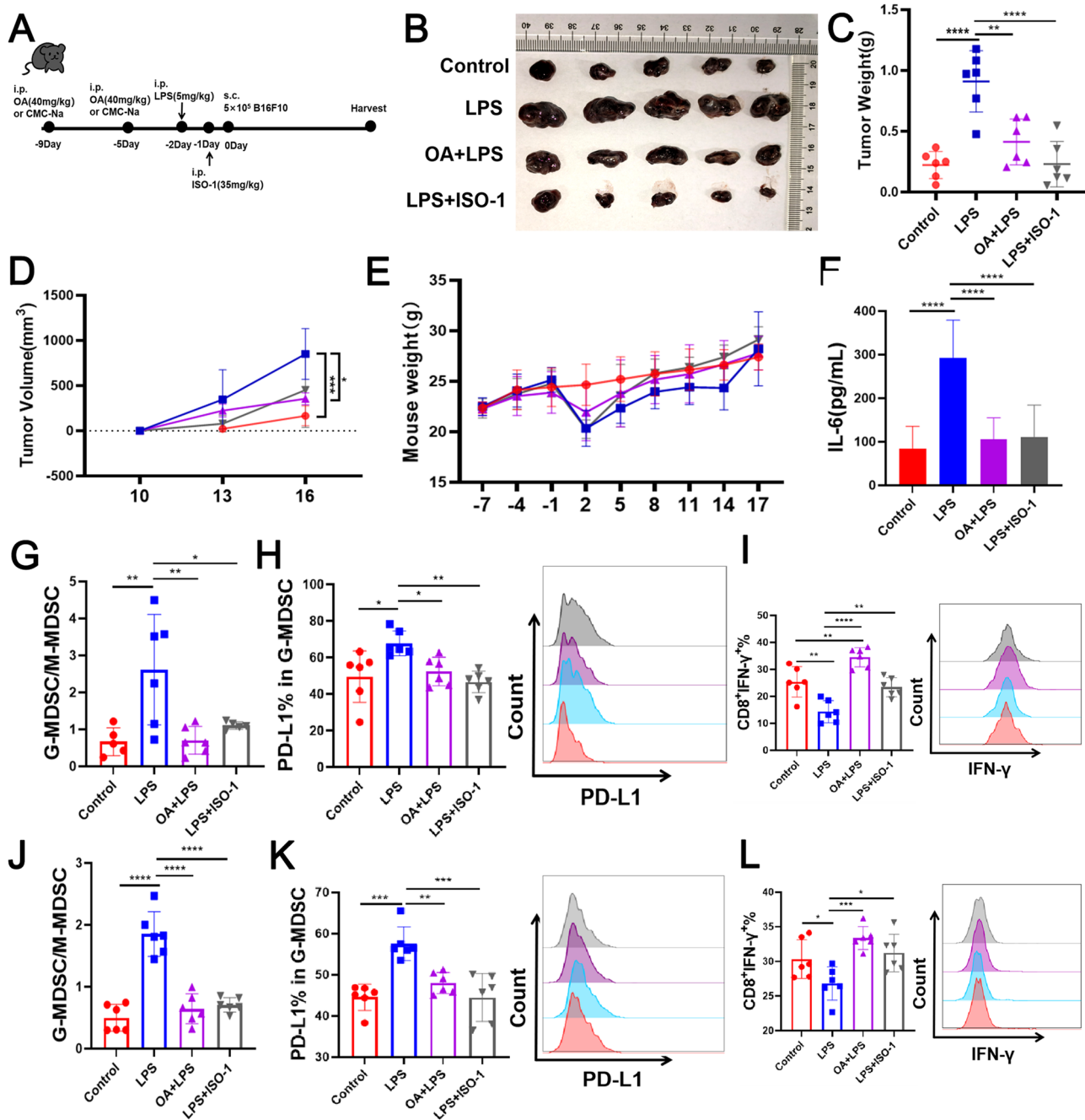


Fig. 8 Sepsis-induced melanoma progression is alleviated by trained immunity/ISO-1. (A) Schematic diagram of the construction of sepsis model ($n=6$). (B) Representative diagrams of melanoma tissues obtained from different groups. (C) Comparison of melanoma weights. (D) Comparison of melanoma growth curves. (E) Curve of body weight changes in mice. (F) The expression level of IL-6 in serum

detected by ELISA. The ratio of (G) G-MDSC/M-MDSC, (H) PD-L1⁺G-MDSC or (I) CD8⁺IFN- γ ⁺T cells in tumor tissue were detected FCM. The ratio of (J) G-MDSC/M-MDSC, (K) PD-L1⁺G-MDSC or (L) CD8⁺IFN- γ ⁺T cells in spleen were detected FCM. The measurement data are presented as the means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

the trained immunity group were lower than those in the sepsis group.

In addition, our results showed that accelerated melanoma progression after sepsis was associated with high serum level of IL-6 in mice. IL-6 appears to be a complex cytokine that is an inflammatory factor during the cytokine

storm associated with sepsis. The prognosis of sepsis survivors can be predicted by serum levels of IL-6 at hospital discharge, which is associated with the risk of cardiovascular disease, new-onset cancer, and mortality [46]. It has been reported that increased serum level of IL-6 in melanoma patients may imply greater drug resistance and poorer

survival rates [47]. IL-6 promotes melanoma progression by activating p38 α -MAPK and upregulating WNT5A expression [48]. IL-6 also promotes melanoma progression by activating MDSCs via STAT5, upregulating CCR5 in MDSCs, and increasing their immunosuppressive capacity [49].

MIF, a key upstream factor of innate immunity, is rapidly secreted upon stimulation by foreign antigens, but the overexpression of MIF has been associated with a variety of immune diseases and cancers [50]. MIF is a potential prognostic marker and therapeutic target in sepsis [36]. Its overexpression leads to the proliferation of G-MDSCs and the upregulation of PD-L1 expression, increasing the immunosuppressive capacity of G-MDSCs [51]. Moreover, MIF also induces IL-6 expression [52], which further enhances the proliferation of G-MDSCs and tumor cells [53]. After molecular docking, we found that there was an active binding site for OA and MIF and observed a decrease in MIF expression during OA-induced trained immunity. In most solid tumors and hematological cancers, patients exhibit high expression and secretion of MIF, which often also implies a poor prognosis [54]. Deletion of MIF inhibits acute colitis-associated colorectal cancer, suggesting that MIF may be a key target of inflammation-promoted cancer [55].

MIF promotes IL-6 secretion by bone marrow-derived mesenchymal stromal cells, which is reversed by the addition of ISO-1 (a MIF inhibitor) [52]. Hypoxia induces the reprogramming of lipid metabolism in laryngeal cancer cells via the MIF/IL-6/JAK-STAT pathway, and treatment of mice with ISO-1 attenuates hormonal conditions and reduces serum IL-6 levels in mice [56]. Therefore, we hypothesized that MIF may be an upstream target of IL-6, stimulating the secretion of IL-6 in the body. In our study, elevated MIF expression was detected in our sepsis-melanoma model. However, this phenomenon was effectively counteracted by OA-induced trained immunity. ISO-1 reduced the serum IL-6 concentration and decreased the number of G-MDSCs/M-MDSCs and decreased the expression of PD-L1 on G-MDSCs, thus increasing the ability of CD8⁺ T cells to secrete IFN- γ , which were similar to the state observed in the trained immunity group. Nevertheless, ISO-1 treatment failed to reverse the decline in body weight caused by sepsis, whereas OA-induced trained immunity effectively safeguarded septic mice, further emphasizing the superiority of trained immunity-based treatment for sepsis. Our data suggest that OA-induced trained immunity may reverse post-sepsis immunoparalysis in mice by targeting MIF, as indicated by the decrease in the serum level of IL-6 and the ratio of PD-L1⁺G-MDSCs and the increase in the number of CD8⁺IFN- γ ⁺T cells. Therefore, it restrained the progression of melanoma.

Danahy et al. reported that the number of weeks/months of immunoparalysis following sepsis could promote melanoma progression. This progression might be related to the post-sepsis environment reducing the infiltration of CD8⁺T cells within the tumor, decreasing the proliferation of CD8⁺T cells and decreasing the expression of activating receptors such as PD-1 and LAG-3, which can be significantly alleviated by anti-PD-L1 therapy [14]. However, that article did not propose an upstream process for this phenomenon and did not suggest safe treatment strategies. Our study, based on the data of Danahy et al., further explored the mechanism underlying this phenomenon and provide new strategies for preventing or treating sepsis-complicated cancer.

And the friend-foe relationship between sepsis and tumors seems to be related to when they occur. Another report by Danahy et al. demonstrated that the induction of sepsis in tumor-bearing mice inhibited tumor progression and prolonged survival [57]. The authors attributed this to the fact that sepsis reactivates dormant CD8⁺T cells and prolongs survival when it occurs in hosts with small, poorly developed tumors. This highlights the intricate interplay between inflammation and cancer, which warrants further investigation. This duality underscores the need for stage-specific interventions in sepsis-associated cancer management.

Our study underscores the phenomenon of increased cancer incidence during the immunoparalysis phase of sepsis. This implies that in clinical practice, the focus should extend beyond the hyperinflammatory phase of sepsis to include vigilant monitoring of patients' conditions during the sepsis-induced immunosuppressive period. Timely intervention during this phase is crucial for preventing secondary infections and complications. Here, we provide evidence that sepsis models exhibit immunoparalysis, which promotes melanoma growth. OA-induced trained immunity, by targeting MIF, has the potential to reverse this phenomenon in sepsis-induced immunoparalysis.

5 Conclusion

Our study demonstrates that sepsis-induced immunoparalysis establishes a tumor-permissive microenvironment by orchestrating immunosuppressive cascades: elevated IL-6 drives MDSCs expansion and PD-L1 upregulation, while CD8⁺T cell depletion and functional impairment collectively accelerate melanoma progression. Crucially, we identify OA as a potent inducer of trained immunity in macrophages, which reverses immunoparalysis through dual targeting of the MIF-IL-6 axis. By suppressing MIF expression, OA reduces IL-6 secretion, normalizes the G-MDSCs/M-MDSCs ratio, and restores CD8⁺T

cell-mediated antitumor immunity, thereby mitigating sepsis-associated tumor growth. In summary, this work bridges the gap between sepsis immunology and oncology, providing a mechanistic foundation for preventing cancer complications in sepsis survivors through immune reprogramming.

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Author contributions Y.L. performed and analyzed all the experiments and wrote the manuscript. D.Y., L.R., L.J. and W.J. participated in the animal experiments. H.Y. and D.H. conceived and designed the project. H.Y. and Z.G. codesigned experiments and co-wrote the manuscript. All authors reviewed and approved the manuscript final version. All data were generated in-house. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics statement The animal study was reviewed and approved by the Animal Protection and Ethics Committee of Nanjing University (IACUC-D2202077).

Compliance with ethics requirements All experiments involving animals were conducted according to the ethical policies and procedures approved by the Animal Protection and Ethics Committee of Nanjing University (IACUC-D2202077).

Consent for publication The manuscript has been approved by all authors for publication.

Clinical trial number Not applicable.

Competing interests The authors declare no competing interests.

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