Phenotypic comparison and the potential antitumor function of immortalized bone marrow-derived macrophages (iBMDMs)

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Abstract

Macrophages are an important component of innate immunity and involved in the immune regulation of multiple diseases. The functional diversity and plasticity make macrophages to exhibit different polarization phenotypes after different stimuli. During tumor progression, the M2-like polarized tumor-associated macrophages (TAMs) promote tumor progression by assisting immune escape, facilitating tumor cell metastasis, and switching tumor angiogenesis. Our previous studies demonstrated that functional remodeling of TAMs through engineered-modifying or gene-editing provides the potential immunotherapy for tumor. However, lack of proliferation capacity and maintained immune memory of infused macrophages restricts the application of macrophage-based therapeutic strategies in the repressive tumor immune microenvironment (TIME). Although J2 retrovirus infection enabled immortalization of bone marrow-derived macrophages (iBMDMs) and facilitated the mechanisms exploration and application, little is known about the phenotypic and functional differences among multi kinds of macrophages. In this study, we demonstrated iBMDMs exhibited the features of rapid proliferation and long-term survival. We also compared iBMDMs with RAW264.7 cell line and mouse primary BMDMs with in vitro and in vivo experiments, indicating that the iBMDMs could undergo the same polarization response as normal macrophages with no obvious cellular morphology changes after polarization. What's more, iBMDMs owned stronger phagocytosis and pro-apoptosis functions on tumor cells. In addition, M1-polarized iBMDMs could maintain the anti-tumor phenotypes and domesticated the recruited macrophages of receptor mice, which further improved the TIME and repressed tumor growth. In summary, iBMDMs can serve as a good object for the function and mechanism study of macrophages and the optional source of macrophage immunotherapy.

Introduction

Macrophages are an important component of intrinsic immunity and possess a variety of functions, including homeostasis maintenance, removal of cellular debris, elimination of pathogens and modulation of inflammatory responses[1, 2]. In the tumor microenvironment, tumor-associated macrophages (TAMs) participate in immune regulation and tumor angiogenesis to affect tumor development[3–5]. Due to the different stimuli in the microenvironment, TAMs present "two-sided" roles with various polarized phenotypes. Macrophages can be activated by interferon gamma (IFNγ) and Toll-like receptor (TLR) agonists to develop an inflammatory (M1-like) phenotype, thus exhibiting proinflammatory characteristics with microbial killing and tumor growth inhibition[6, 7]. Conversely, in response to interleukin-4 (IL-4), IL-13 and IL-10 (M2-like activation), macrophages release anti-inflammatory factors, which promote immunosuppression, debris removal, angiogenesis, tissue remodeling and repair[7–9]. Investigating the complex cellular mechanisms of macrophages in the context of disease is emerging as a fundamental step in understanding pathogenesis as well as performing macrophage immunotherapy[10].

Considering the pivotal influence of macrophage development and function on disease progression, immunotherapy based on macrophages has achieved some progress in recent years[11–15]. Our
previous studies have demonstrated that stimulated M1 macrophages and miR-125a-overexpressing macrophages could alleviate liver fibrosis and repress tumor growth, respectively[16]. The strong plasticity and functional diversity endow macrophages with better immunotherapeutic effects and advantages. However, macrophage-based therapeutic strategies still face two limitations in terms of antitumor immunity. On the one hand, although engineered modified or gene-edited macrophages exhibit obvious antitumor potential, the repressive tumor immune microenvironment (TIME) accelerates their functional remodeling to limit immunotherapy. On the other hand, the lack of proliferation ability of infused macrophages makes gene editing and cell harvesting more difficult, which increases the treatment time and immunotherapy cost[17]. How to solve the problem of microenvironment domestication and lasting proliferation ability has become the focus of macrophage immunotherapy.

Currently, the majority of macrophage sources for basic research and immunotherapy exploration consist of bone marrow-derived macrophages (BMDMs)[17–19], induced pluripotent stem cell-derived macrophages (iPS-Mϕ)[20–22] and the RAW264.7 cell line[23–25]. BMDMs are fully developed and function regulable and are more suitable for in vitro experiments and in vivo verification. However, the BMDMs could not achieve stable genotypic transformation due to proliferation limitations. Flexible gene editing and functional modification are advantages of iPS-Mϕs in cellular immunotherapy. It is extremely costly and difficult to obtain and culture iPS-Mϕs. Meanwhile, it has been reported that iPS-Mϕs present an M2-like polarization phenotype, which is not appropriate for tumor immunotherapy[26]. RAW264.7 is a kind of fusion-immortalized monocyte-macrophage line of BALB/c mouse origin that was established from murine tumors induced with Abelson leukemia virus by Raschke et al. in 1978[27]. The RAW264.7 cells were only used for some in vitro experiments of macrophage function analysis[28]. Therefore, it is crucial to seek effective and safe cell sources for macrophage immunotherapy.

A growing amount of evidence highlights the intriguing possibility that macrophage immortalization may be a viable strategy for macrophage-based immunotherapy. J2 retrovirus infection-enabled immortalization has been successfully applied to fetal liver macrophages, spleen macrophages, microglia, and bone marrow-derived macrophages (BMDMs)[29–32]. Immortalized macrophages express surface biomarkers of macrophages and possess typical functional characteristics. In addition, they share strong proliferation ability and long-term survival potential. Therefore, gene-edited immortalized macrophages are easy to construct, which facilitates the advancement of macrophage regulatory mechanisms. The study by Iolanda Spera et al. in 2021 detected and analyzed the functions of the immortalized BMDM (iBMDM) cell lineage from a metabolic point of view[33]. By determining intracellular and extracellular metabolites as well as the phenotypic characteristics of immortalized versus primary BMDMs, it was concluded that immortalized BMDMs exhibited similar metabolism and polarization characteristics under both classical and alternative stimulation. However, no study has systematically evaluated and compared the biosafety, immunological characteristics and antitumor functions of iBMDMs. In this study, we detected the proliferation efficiency and survival time of iBMDMs both in vitro and in vivo, indicating that iBMDMs have good biosafety and low immunogenicity. Immunology tests and coculture experiments with tumor cells were used to analyze the effect of iBMDMs on the malignant biological behaviors of tumor cells. Finally, the infusions of different macrophages into
tumor-bearing mice suggested that iBMDMs present even stronger antitumor potential than primary BMDMs. Our study comprehensively explores the antitumor functions of iBMDMs in vitro and in vivo and demonstrates that iBMDMs are an optional source of macrophage immunotherapy.

Materials and Methods

Animals and tumor models

Wild-type C57BL/6 mice used in this study were maintained in a specific pathogen-free facility. All the animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University to ensure the ethical and humane treatment of the animals. And all experiments used 8-week-old to 12-week-old male mice. The LLC cell line was purchased from the authenticated ATCC repository in 2014. LLC was mixed with macrophages at a ratio of 5:1 ($5 \times 10^6:1 \times 10^6$) and injected into the subcutaneous tissue of the backs of mice. The length and width of the tumor tissue were measured using a ruler and analyzed after 3 weeks of coculture. The mice were sacrificed at 2 or 3 weeks after inoculation, and tumors were digested to a single cell suspension with type V collagenase (Sigma, St. Louis, MO) and DNase I (Roche, Basel, Switzerland) for flow cytometry.

Cell culture

iBMDMs, RAW264.7 cells, and LLC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS). BMDMs were extracted from the bone marrow of C57BL/6 mice and cultured in DMEM supplemented with 10% FCS and 25 ng/mL murine macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ) for 7 days, and flow cytometry analysis was used to detect the stimulation efficiency of BMDMs. The GFP fragment was inserted into the viral vector and then infected into iBMDM to construct a stable cell line for subsequent experiments. In polarization-related experiments, macrophages were stimulated with IFN-γ (20 ng/mL, PeproTech), LPS (50 ng/mL, Sigma) or IL4 (20 ng/mL, PeproTech) for 24 h and then used in follow-up experiments.

Immunofluorescence

The slides were placed into a 12-well plate and coated with polylysine, and the macrophages were plated on the slide to adhere to the wall, stimulated with IFN-γ, LPS or IL4 for 24 h, and stained with anti-iNOS and anti-ARG1 (CST, Danvers, MA). Mouse subcutaneous tumor tissues were removed and fixed in 4% paraformaldehyde, and then 30% sucrose solution was used to dehydrate them. The tissues were embedded and frozen for sectioning. The sections were stained with anti-F4/80 (Invitrogen), anti-iNOS or anti-MR and photographed with a fluorescence microscope (M5000, Thermo, Waltham, MA).

Flow cytometry

Tumor tissue was removed from the subcutaneous skin of mice, cut up, digested with 1 mg/mL collagenase V (Sigma) and 4 mg/mL DNase I (Roche) at 37°C for 30 min to make a cell suspension,
which was filtered through a 70-micron filter membrane and stained with flow cytometry antibody. Dead cells were removed by 7AAD. All the experimental results were analyzed by FACSCalibur and FACSCanto flow cytometry (BD Immunocytometry Systems). Data were processed by FlowJo v10 software (FlowJo, LLC, Ashland, OR).

**Phagocytosis**

LLC cell lines were suspended in PBS containing 0.1% serum at a concentration of 10^6 cells/ml, and the final concentration was 5 nM carboxyfluorescein succinimidyl amino ester (CFSE; MCE, NJ). The cells were stained at room temperature and shielded from light for 7 min. After staining, the same volume of serum was used to terminate the staining, and the stained LLC cells were incubated with macrophages at a ratio of 2:1 for 1 h. The phagocytosis ratio was detected by FACSCanto staining with anti-F4/80 (Invitrogen). The strength of phagocytosis between different cell lines was compared based on the percentage of double-positive cells in the flow-through results.

**Apoptosis**

Macrophages were stimulated with IFN-γ, LPS or IL4 for 24 h, the supernatant of the stimulated cells was taken. Tumor cells were seeded in 12-well plates, incubated with macrophage supernatant for 48 hours, stained with an Annexin V apoptosis detection kit (Invitrogen) and detected using FACS Calibur flow cytometry. Effects of macrophage supernatants with different polarization states on early apoptosis, mid-apoptosis, and late apoptosis of tumor cells analyzed by flow data analysis.

**Cell proliferation**

The LLC cell line was seeded in 12-well plates, macrophage supernatants of different stimulation states were taken and incubated with tumor cells in LLC for 24 h. After fixation with 4% paraformaldehyde and staining with crystal violet (Kehao, Xi’an, China), the supernatants were washed with PBS 3 times and resuspended with acetic acid, the absorbance of the liquids was measured by an enzyme marker, and the effect of macrophage supernatants of each polarization state on the proliferation of tumor cells was compared based on the strength of the absorbance.

**Cell migration**

A marker was used to draw three lines on the back of the 12-well plate, and the LLC cells were inoculated into the 12-well plate. When the cells grew to 80%, the tip of the gun was used to draw a straight line perpendicular to the three lines, macrophage supernatants of different polarization states were added, photos of the scratch were taken at the intersection of the 3 straight lines according to different times, and the cell migration area was counted.

**RT–PCR**

Total cellular and tissue RNA was extracted with TRIzol (Invitrogen) reagent according to the instructions, and the concentration of the extracted RNA was determined and then transcribed into CDNA using a
reverse transcription kit (Yeasen, Shanghai, China). SYBR Premix EX Taq (Yeasen) was added to the system according to its instructions, and real-time quantitative PCR was performed by QuantStudio.

Statistics

All experimental data were statistically processed by GraphPad Prism 5 software, and unpaired Student’s t tests or one-way ANOVA was used for comparison. When the data results were expressed as P < 0.05, they were considered statistically significant.

Results

iBMDMs exhibit the similar cellular characteristics of primary macrophage

CD11b and F4/80 are specific markers for macrophages[34]. To identify the macrophage characteristics of iBMDMs, we performed FACS analysis by anti-F4/80 and anti-CD11b staining. As expected, iBMDMs, BMDMs and RAW264.7 cells shared similar macrophage biomarker expression patterns (Fig. 1a). For further morphological comparison, the three kinds of macrophages were stimulated with different cytokines for observation by microscopy. The M1-polarized BMDMs had some appearing cogwheel, while M2-polarized BMDMs had longer pseudopodia compared with controls. The RAW264.7 cells protruded more pseudopodia after polarization stimulation. In contrast to polarized BMDMs or RAW264.7 cells, iBMDMs presented polygon or round shapes regardless of the cytokines added (Fig. 1b).

Next, we verified the survival and biosafety of iBMDMs in vivo and in vitro. In vitro, we cultured the cells for a long time and passaged them at a ratio of 1:10 each time. According to the passage cycle, the proliferation activity of the cells was measured. The results showed that the proliferation efficiency of iBMDMs began to slow down after 3 weeks of in vitro culture and was almost quiescent after 6 weeks (Fig. 1c). Similarly, we validated the survival cycle of iBMDMs in mice. iBMDMs survived for 3 weeks, but the number of surviving iBMDMs gradually decreased over time (Fig. 1d). The results suggested that iBMDMs possessed a long-term lifespan but no immortalization capacity both in vivo and in vitro, which provided feasibility for iBMDM-based cell 2therapy. To ensure the biosafety of iBMDMs, we carried out HE staining by using sections of different tissues from iBMDM reinfused mice. The results showed that iBMDMs were nontoxic to mouse tissue and can be used for subsequent treatment in mice (Fig. 1e). The above data suggested that iBMDMs maintained rapid proliferation and long-term lifespan, indicating potential cell sources for immunotherapy.

Normal polarization response is possible with iBMDMs

Phenotypic alterations in iBMDMs were assessed by qPCR detection of specific M1 (IL-1β and iNOS) and M2 (Arg1 and MR) polarization biomarkers. The results revealed that after LPS + INF-γ treatment, the levels of M1 genes were specifically increased in all three types of macrophages. In addition, the expression of M2 polarization markers was obviously increased in IL-4-treated macrophages. It should be
noted that although the iBMDMs presented a similar polarization response to primary BMDMs or macrophage lines, the mRNA elevations of all M1-specific biomarkers (IL-1β, iNOS and TNF-α) were mild in iBMDMs compared with RAW264.7 cells or BMDMs (Fig. 2a-c). A similar conclusion was verified by ELISA, suggesting that M1-iBMDMs produced much higher levels of proinflammatory cytokines (TNF-α and IL-12) and lower levels of anti-inflammatory factors (IL-10 and TGF-β). Interestingly, the differences in secreted proteins between the three kinds of macrophages were not that sharp. particularly significant.

Under both M1- and M2-polarization conditions, iBMDMs presented the same level of inflammatory response as BMDMs (Fig. 2d). M1-type macrophages have elevated aerobic glycolysis and produce inducible nitric oxide synthase (iNOS), which is associated with antitumor and anti-infection immunity[35]. ARG1 is an enzyme involved in arginine metabolism and generation in macrophages that leads to T-cell exhaustion and functional repression[36]. iNOS and ARG1 are essential markers of M1 polarization and M2 polarization, respectively. To further observe the expression of polarization markers in different cell lines, three kinds of macrophages were stimulated and stained with anti-iNOS and anti-ARG1. The immunofluorescence results showed a similar conclusion that M1-iBMDMs expressed higher levels of iNOS, while M2-iBMDMs exhibited advantages in ARG1 expression, which was even more obvious than that of BMDMs and RAW264.7 cells (Fig. 2e-g). These data suggested that iBMDMs perform a similar polarization response as other macrophage sources.

**iBMDMs strongly phagocytose tumor cells**

Next, we explored the phagocytosis function of macrophages, which plays a key role in tumor killing and pathogen removal[37]. To examine the differences in phagocytosis among the three kinds of macrophages, iBMDMs, RAW264.7 cells and BMDMs were stimulated with polarization factors for 24 h and then cocultured with CFSE-stained LLC cells at a ratio of 1:2. Two hours later, the phagocytosis capacity was evaluated by calculating the proportion of macrophages swallowing tumor cells (F4/80*CFSE+) using flow cytometry. It should be noted that the phagocytic ability of M1-BMDMs was elevated 10-fold compared with that of quiescent BMDMs, which is equivalent to the response level of RAW264.7 cells. However, after LPS + INF-γ treatment, iBMDMs presented much stronger phagocytic enhancement. The engulfment rate of M1-loaded iBMDMs was almost 20 times that of quiescent iBMDMs (Fig. 3a-d). In summary, M1-iBMDMs exhibited strong phagocytosis, which was stronger than that of BMDMs and RAW264.7 cells, and the results further demonstrated that immortalized BMDMs could interact well with tumor cells.

**The paracrine of iBMDMs inhibits proliferation and promotes apoptosis of tumor cells**

In addition to direct phagocytosis to inhibit tumor progression, macrophages can also modulate tumor cell migration, proliferation and apoptosis by secreting multiple cytokines and inflammatory mediators. To further verify the macrophage function of iBMDMs, we incubated LLC cells in the supernatant of macrophages with different treatments for 24 hours and determined cell proliferation by measuring
absorbance after staining with crystal violet. As expected, the supernatant of all three groups of macrophages exhibited the inhibition of tumor cell proliferation, especially that of M1 macrophages. The results were consistent with previous results showing that M1-type macrophages have the ability to inhibit tumor growth. Interestingly, compared with RAW264.7 cells and BMDMs, iBMDMs showed a significant decrease in tumor cell proliferation after M1 polarization. This result proved that the iBMDMs had a stronger antitumor function (Fig. 4a).

Furthermore, we cocultured LLC cells with macrophage supernatant for 48 hours and detected LLC apoptosis by using Annexin V/PI staining. We found that the supernatant of M1 macrophages promoted tumor cell apoptosis more obviously than PBS-treated macrophages. In addition, although the iBMDM supernatant displayed a certain ability to induce tumor cell apoptosis, the percentage of late apoptotic cells was much lower than that of LLC cells incubated with supernatant from BMDMs or RAW264.7 cells (Fig. 4b and 4c). We speculate that the factors secreted by iBMDMs mainly influence the early stage of tumor cell apoptosis. In summary, the secreted component of iBMDMs had obvious effects on inhibiting tumor cell proliferation and promoting apoptosis, which indicated that iBMDMs could also repress tumor growth indirectly.

**iBMDMs repress tumor cell migration via inhibiting EMT progress**

Modulating tumor cell invasion and participating in the formation of migrated units are important functions of tumor-associated macrophages. The wound healing assay was performed by incubating tumor cells with macrophage supernatants to test the cell motion at different time periods. The results showed that the modulation effect was not obvious after 16 h of incubation. However, tumor migration was significantly inhibited by M1 macrophage supernatant after 24 h treatment. As expected, iBMDMs, especially M1-iBMDMs, presented the most remarkable inhibitory effect (Fig. 5a-e).

Multiple factors can influence tumor cell infiltration and metastasis. To investigate the mechanisms by which macrophage paracrine signaling affects LLC mobility, we cocultured macrophage supernatants with LLC cells for 24 h and then detected the expression of EMT (epithelial-mesenchymal transition) -related genes, which are responsible for tumor cell migration to some extent[38]. The data suggested that iBMDMs exhibited notable repressive effects on LLC EMT. After administration of M1-iBMDM supernatant, the expression of the tight junction-related membrane protein ZO-1 and the EMT essential transcription factors Snail1 and Twist was greatly reduced, which was superior to the other two types of macrophages (Fig. 5f). The above results demonstrated that the paracrine pathway of iBMDMs plays a significant role in tumor cell EMT progression and migration regulation.

**M1-polarized iBMDMs rather than primary BMDMs repress tumor growth in vivo**

Previous experiments have demonstrated the macrophage characteristics and antitumor functions of iBMDMs in vitro. To verify the phenotypes and effects of iBMDMs during tumor progression, we
stimulated EGFP-modified BMDMs or iBMDMs into M1 polarization and mixed them with LLC at a ratio of 1:5 to inoculate them subcutaneously on the backs of mice. The tumor size and weight were monitored after 3 weeks. The tumor volume and weight in the M1-iBMDM group were smaller than those in the M0-iBMDM group, which had an inhibitory effect on tumor growth. Compared with BMDMs, M1-BMDMs did not have a significant inhibitory effect and even showed an upward trend (Fig. 6a, 6b). The results showed that the tumor size and weight of the iBMDM group treated with M1 polarization were significantly decreased compared with those of the control group (M0 group) (Fig. 6A). Meanwhile, the changes in tumor size and weight in the BMDM infusion groups were not obvious and were even increased in the M1-BMDM treatment group (Fig. 6B). Ki67 and TUNEL staining was also performed using tumor sections. The Ki67 staining data suggested that there was no significant change between the M1-BMDM and M0-BMDM groups. In contrast, M1-iBMDM treatment inhibited tumor cell proliferation and reduced tumor malignancy (Fig. 6c). Moreover, the TUNEL staining results showed that iBMDMs could dramatically promote apoptosis in tumors compared with BMDMs (Fig. 6d). These results indicated that M1-polarized iBMDMs possessed significant antitumor activity in vivo by modulating the malignant biological behaviors of tumor cells.

Next, we examined the expression of representative molecular markers of different polarization phenotypes and macrophage function with tumor sections. The immunofluorescence results showed that there were significantly more M2-type macrophages (MR$^{+}$ F4/80$^{+}$) than M1-type macrophages (iNOS$^{+}$ F4/80$^{+}$) in the tumor tissues of both the iBMDM and BMDM groups. However, regardless of M0- and M1-polarized BMDM therapy, few iNOS-positive macrophages were detected after three weeks, suggesting that BMDM infusion did not domesticate the recruited macrophages. This might be because the survival time of infused BMDMs was too short to exert the immune regulatory function completely. In contrast, iBMDM infusion stimulated more M1-like TAMs to improve the immune microenvironment and repress tumor growth. Especially after the infusion of M1-type iBMDMs, the number of iNOS-positive TAMs increased significantly (Fig. 6e). Most infused macrophages could not last their lifespan to 3 weeks in tumors. Even long-term iBMDMs could not be detected in the infused tissue in vivo (Fig. 1). Obviously, the infused iBMDMs had a profound and sustained impact on the endogenous recruited macrophages and tumor microenvironment. The effect of switching TAMs into the M1-like phenotype might be the reason why M1-iBMDM therapy could inhibit tumor development.

**M1-polarized iBMDMs domesticate self-recruited TAMs and improve the tumor microenvironment**

To further investigate the impact of iBMDMs on the tumor microenvironment, we digested the tumor tissues into single-cell suspensions for further FACS analysis. TAMs were classified into three subgroups based on Ly6C and major histocompatibility complex class II (MHCII class) expression: mature TAMs (ma-TAMs) (Ly6C$^{-}$MHCII$^{+}$), immature TAMs (imm-TAMs) (Ly6C$^{-}$MHCII$^{-}$), and TAM precursors (pre-TAMs) (Ly6C$^{+}$MHCII$^{-}$)[39, 40]. Many studies have demonstrated that mature TAMs highly express M1 polarization-related markers and exert antitumor functions. Compared to that in the BMDM group, the proportion of mature TAMs in the iBMDM group was significantly increased, while the proportion of
immature TAMs and TAM precursors was significantly decreased. Similarly, the M1 iBMDM group had a significantly higher population of Ly6C<sup>−</sup>MHCII<sup>+</sup> ma-TAMs than the M0-iBMDM treatment group, while BMDMs did not have this result (Fig. 7a and 7b).

G-MDSCs and M-MDSCs play an important role in the tumor microenvironment. In tumor-related myeloid cells (CD11b<sup>+</sup>), the proportions of G-MDSCs and M-MDSCs in the iBMDM group were lower than those in the BMDM group. iBMDMs can reduce G-MDSCs and M-MDSCs in the tumor microenvironment, improve the tumor microenvironment, and inhibit tumor growth. Moreover, M1 iBMDMs had a more significant inhibitory effect on M-MDSCs (Fig. 7c and 7d). In addition, iBMDMs can promote the generation of CD8<sup>+</sup> T cells in tumors, increase the direct killing of tumor cells (Fig. 7e and 7f).

In summary, iBMDMs not only have excellent macrophage function in vitro but also improve the tumor microenvironment in vivo, recruiting endogenous macrophages to exhibit an antitumor phenotype while reducing the proliferation of various tumor-promoting immune cells and increasing the number of antitumor CD8<sup>+</sup> T cells, thus inhibiting tumor growth. In vivo, the therapeutic effect of iBMDMs is superior to that of BMDMs.

**Discussion**

Macrophages are considered as the key immune regulator during tumor initiation and development. Macrophages not only contribute to the recruitment and activation of immune cells in the tumor microenvironment (TME) but also play an important role in tumor cell metastasis[41]. Targeting tumor-associated macrophages (TAMs) is currently considered a promising strategy for combating cancer[42]. How to polarize TAMs to an antitumor state without affecting macrophage activity is of great research significance to reduce tumor growth and metastasis. The current understanding of the mechanisms involved in controlling the cancer and metastatic cascade response remains limited. Studies of mechanisms that regulate innate immune activation require in vitro cellular experiments or in vivo therapeutic validation. Currently, the most common used cell resources for macrophage research are BMDMs and RAW264.7 cells[28]. BMDMs, as primary bone marrow-derived cells, need to be obtained from the bone marrow followed by stimulation and cultivation with different cytokines, which requires much more time and cost. Meanwhile, the short survival time of BMDMs is not suitable for establishing a stable transduced cell line, which limits their application for in vivo transfusion therapy[43]. In contrast, RAW264.7 is a type of peritoneal macrophage from confluent mice that has immunogenicity and tumor-promoting properties and is not suitable for in vivo reperfusion therapy. Immortalized macrophages may help meet the current needs of reducing macrophage research costs and establishing stable and transmissible cell lines.

This study introduces the iBMDM cell line constructed by Elisabetta Blasi et al. in 1986[29] and compares it with BMDM and RAW264.7 cell lines in terms of phenotype, characteristics, polarization detection, and in vitro and in vivo antitumor functions, proving the feasibility of using iBMDM cell lines as macrophages for research within a certain range. Previous studies have not systematically compared the differences in
function and characteristics between immortalized macrophages and nonimmortalized cells and explored their advantages and disadvantages as macrophage therapy. This experiment validated the relative safety and effectiveness of using iBMDMs as a macrophage therapy resource. We also investigated macrophage polarization regulation and tumor microenvironment domestication reversal, especially the influence on endogenously recruited macrophages. Our data indicate that the iBMDM cell line is actually not immortalized but possesses a relatively longer survival time both in vitro and in vivo. The long-term lifespan of iBMDMs provides more possibilities for cell therapy while ensuring biological safety because they do not remain in the body after reinfusion. In addition, we also demonstrated that although iBMDMs exhibited similar characteristics as other macrophages (BMDMs and RAW264.7 cells), certain differences were found among different macrophage sources in the expression levels of polarization markers and their in vitro and in vivo antitumor functions. It should be noted that iBMDMs were demonstrated to exhibit a superior ability to improve the tumor microenvironment and repress tumor development compared to BMDMs.

In conclusion, our data indicate that these long-term iBMDMs possess macrophage characteristics and functions and are superior to other macrophages in some aspects. It can be used for in vivo and in vitro experiments on macrophages and is expected to serve as a cell resource for macrophage reinfusion therapy. Moreover, iBMDMs could be further modified with genetic editing. The edited iBMDMs presented a more stable phenotype and stronger antitumor functions. Therefore, iBMDMs have great potential for application in immune cell therapy. We also hope to further investigate the molecular mechanisms of the differences between BMDMs and iBMDMs. To advance research on macrophage therapy and its clinical application as soon as possible, more comprehensive and in-depth research needs to be implemented to identify the key molecules involved in phenotypic and functional changes. The revelation of the immortalization characteristics of macrophages also provides a reference for the immortalization of other immune cells (such as NK cells, dendritic cells, and T cells). and lays a foundation for further improving tumor immunotherapy.

**Declarations**

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This study was performed in the Graduates Innovation Center of Fourth Military Medical University.

**Conflict of Interest**

No potential conflicts of interest were disclosed.
Author Contributions


Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

This research did not require ethical approval because this study did not contain any individual person’s data in any form.

References


Figures

**Figure 1**

**iBDMs exhibit the similar cellular characteristics of primary macrophage.** a Flow cytometry was used to detect macrophage-specific markers after staining with F4/80 and CD11b for iBDMs, RAW264.7 cells, and BMDMs. b Using PBS, LPS+IFN-γ- and IL-4-stimulated iBDMs, RAW264.7 cells, and BMDMs,
morphological changes in macrophages were observed after polarization stimulation using an inverted microscope. c Pass iBMDMs at a ratio of 1:10. An inverted microscope was used to take photos and observe morphological changes. Determine cell proliferation activity in vitro based on the length of its passage cycle. d iBMDMs were injected into mice, and fluorescence signal expression at different times was detected to determine the survival time of iBMDMs in mice. e Injection of ibmdm into mice and detection of the biological safety of iBMDMs in mice by HE staining of mouse tissues. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Normal polarization response is possible with iBMDM. a-g iBMDMs, RAW264.7 cells and BMDMs were treated with PBS, LPS+IFN-γ and IL4 for 24 hours. a-c QPCR was used to detect the expression of M1 polarization markers (IL-1β and iNOS) and M2 polarization markers (MR and ARG1) in iBMDMs, RAW264.7 cells, and BMDMs under different stimuli. d Macrophage supernatant was collected under different stimuli, and ELISA was used to detect the expression of M1 markers (TNF-α and IL-12) and M2 markers (IL10 and TGF-β) in the macrophage supernatant. e-g Anti-iNOS and anti-ARG1 were used as primary antibodies for immunofluorescence staining and to detect the expression of markers after macrophage polarization. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 3

iBMDMs strongly phagocytose tumor cells. a-d iBMDMs, RAW264.7 cells and BMDMs were treated with PBS, LPS+IFN-γ and IL4 for 24 hours. Differently treated iBMDMs, RAW264.7 cells, and BMDMs were
coincubated with LLCs at a ratio of 1:2 for one hour, and the phagocytosis ability of macrophages was detected by flow cytometry. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4

The paracrine of iBMDMs inhibits proliferation and promotes apoptosis of tumor cells. a-c iBMDMs, RAW264.7 cells and BMDMs were treated with PBS, LPS+IFN-γ and IL4 for 24 hours. a iBMDMs, RAW264.7 cells and BMDM supernatants were coincubated with LLCs in a 96-well plate for 24 h, and the absorbance was measured by an enzyme labeling instrument after crystal violet staining to detect the effect of macrophage supernatants on the proliferation of tumor cells. b-c iBMDM, RAW264.7, and BMDM supernatants were coincubated with LLCs for 48 h, and apoptosis of tumor cells was detected by flow cytometry. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 5

**iBMDMs repress tumor cell migration via inhibiting EMT progress.**

*a-c* PBS, LPS+IFN-γ, IL4-stimulated iBMDMs, RAW264.7 cells, and BMDMs for 24 h. iBMDMs, RAW264.7 cells, and BMDM supernatants with different polarization states were coincubated with LLCs, and the effect of macrophage supernatants on tumor cell migration was detected by scratch.  

*d-e* ImageJ software was used to process the scratch results and generate statistics on the processing results.  

*f* iBMDMs, RAW264.7 cells, and BMDM supernatants were coincubated with tumor cells for 24 h, and the expression of EMT (epithelial-mesenchymal transition)-related genes was detected by QPCR. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 6

M1-polarized iBMDMs rather than primary BMDMs repress tumor growth *in vivo*. a-b iBMDMs and BMDMs were stimulated with PBS or LPS + IFN-γ for 24 h, mixed with LLC at a ratio of 1:5, and inoculated subcutaneously, and the size and weight of the tumors were measured and compared after 3 weeks. c-d Immobilization, embedding, and sectioning of subcutaneous tumor tissue were performed using Ki67 and TUNEL staining. Fluorescence microscopy was used to detect the expression of Ki67 and TUNEL in tumor tissue, and the effects of the two types of macrophages on tumor cell proliferation and apoptosis in the tumor microenvironment were compared. e Perform F4/80, iNOS, and MR staining on subcutaneous tumor sections. Fluorescence microscopy photography was used to detect the impact of
the two types of macrophages on the tumor microenvironment in tumor tissue. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 7

M1-polarized iBMDMs domesticate self-recruited TAMs and improve the tumor microenvironment. a-f PBS, LPS+IFN-γ-stimulated iBMDMs, and BMDMs for 24 h were mixed with LLC at a ratio of 1:5 and then inoculated subcutaneously. After 3 weeks of tumor inoculation, flow cytometry was used to evaluate the tumor immune microenvironment of mice with different treatments, and the percentages of TAMs (a-b), MDSCs (c-d), and T cells (e-f) in the tumor immune microenvironment were detected and analyzed. Bars, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.