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Polarization of M1 tumor associated macrophage promoted by the activation of TLR3 signal pathway

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ABSTRACT

Objective: To investigate the correlation between activation of toll-like receptors 3 (TLR3) signaling pathway and tumor-associated macrophage and its effect on the tumor growth.
Methods: The mice Lewis lung cancer cell lines 3LL and melanoma B16H10 were used to construct the subcutaneous transplantation tumor models and then they were treated with Poly-ICLC. The curative effect was observed and then the T cell and macrophage phenotypes infiltrated in local tumor were detected by flow cytometry. After the *in vitro* culture of mouse bone marrow-derived macrophage, the real-time PCR and western blot were applied to detect the expression of macrophage activation markers and the activation of intracellular signaling pathways.
Results: The survival time of mice with brown tumor treated with Poly-ICLC significantly increased and the tumor growth was inhibited. The ratio of local tumor-infiltrated Treg decreased, while the ratio of $CD8^+$ T cell increased significantly. The macrophages surface CD206 expression was down-regulated while the expression of iNOS increased. The Poly-ICLC could promote the expression of M1 markers (IL-1β, TNF-α and iNOS) in bone marrow-derived macrophage and inhibited the expression of M2 molecules (Arg-1, YM-1 and CD206). The phosphorylation level of downstream p65, TBK1 and IRF3 increased significantly.
Conclusions: The Poly-ICLC can activate the TLR3 downstream signaling pathway to induce a M1 polarization of tumor associated macrophage, thereby inhibiting the tumor growth.

1. Introduction

The tumor associated macrophages (TAM) refer to a kind of macrophages which are migrated and infiltrated in local tumor during the occurrence and development of tumor, and can be brought together with other immune cells, tumor cells, fibroblast and interstitial cytokines to form a tumor immune microenvironment. The TAM can secrete a variety of cytokines and inflammatory mediators which play a key role in the formation of tumor microenvironment and tumor invasion and metastasis. According to the activation type of macrophage, it can be divided to two main types M1 and M2. The M1 has the anti-tumor effect which can be exerted by secreting the proinflammatory factor, while M2 can promote the growth, invasion and metastasis of tumor through the expression of immune inhibitory signal molecule [1]. The function of M1 and M2 macrophages is entirely different in the tumor microenvironment. The clinical data have showed that M2 TAM can infiltrate in a wide variety of tumors and the infiltration number is negatively correlated with the prognosis of patients [2–5]. Therefore, the regulation and control of M2 TAM can be a key factor for improving the prognosis of tumor. This paper aims to investigate the effect of Poly-ICLC on the macrophage polarization and tumor growth.

2. Materials and methods

2.1. Main reagents

The RT-PCR primer was designed and synthesized by Shanghai Sangon Biotechnology Company. The RT-PCR kit and the ordinary PCR kit were purchased from the Takara Company. The mice Lewis lung cancer cell line 3LL and the

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melanoma B16H10 cell were bought from ATCC and the trizol was purchased from Invitrogen. The DMEM Medium (high glucose), antibiotics, L-glutamine and the fetal calf serum were purchased from Gibco. The protease inhibitor and phosphatase inhibitors were collected from Roche. For the use of western blot, the p-p65 (S536), p-TBK1 (S172), p-IRF3 (S396) primary antibodies and horseradish peroxidase-labeled secondary antibodies were bought from CST. The CD45, CD3, CD4, CD8, Foxp3, CD206, F4/80 and iNOS for flow cytometry were all purchased from eBioscience.

2.2. Animal model

All the animals involved in this study were purchased from the Chongqing Medical University Laboratory Animal Center (C57 mice, clean, 8 weeks old and weighing 20 g). The collected cells were digested by using the pancreatic enzymes and the final concentration was adjusted at 1×10^7/mL. After that, the treated cells were placed in the ice until use. The mice were narcotized with 200 L of 0.75% sodium pentobarbital solution per mouse and then were conducted to an inguinal subcutaneous injection of 5×10^5 tumor cells. The mice in treating group were then received an intraperitoneal injection of 50 mg Poly-ICLC at 5th day after the model construction, and a drug administration every other day for four times was conducted. While the mice in control group were also received the same volume injection of PBS and then the tumor size was calculated. The experiment was approved by the Biomedical Ethics Committee of Chongqing Medical University.

2.3. Cell cultivation

The cultivation of mice Lewis lung cancer cell line 3LL and melanoma B16H10 cell was conducted in the DMEM medium contained 10% fetal calf serum, 1% double resistant and 2 mM L-glutamine. Then the cultivated cells were stored in the incubator with 5% CO_2, saturated humidity and at 37 °C for cultivation. The medium was replaced every 2–3 d and the cells were not transferred to a conventional cultivation until the cell density was moderate. The *in vitro* macrophage cultivation was carried out by using the mouse bone marrow, and after that the BALB/c mice were conducted to death by broken neck dislocation and 75% alcohol disinfection, the bilateral tibiofibula was obtained under the aseptic condition. Then the distal insertion of a syringe needle (5 mL injector) contained with 1 mL DMEM was conducted. The DMEM was injected to get the bone marrow cell, and then the collected cells were placed in sterile tube to a repeated stir until that the cells distributed evenly. Then using the ACK to crack red cells and they were filtered by a 200-mesh sieve. After washing by DMEM for 2 times, the cells were resuspended by using L929 cell-conditioned medium (contained with 30% L929 cell 3-d cultured supernatant) and then were inoculated in a petri dish. After 7 d, the adherent cells were removed and the remaining cells were the bone marrow-derived macrophage.

2.4. Flow cytometry

The single-cell suspension of local tumor tissue was obtained from the collagen digestion and after the isolation by Percol, the mononuclear cell was obtained. The steps for surface antigen staining were conducted strictly in accordance with the Best Protocol (eBioscience). At first, they were blocked by using the CD16/32 antibody for 10 min at 4 °C and after that, were washed by PBS to obtain 300 mL PBS heavy suspension. Finally, they were conducted to a detection by using BD flow cytometry. The data were analyzed and drawned by applying the FlowJo software.

2.5. Real-time PCR

After cell collection, the total RNA was extracted by trizol method and was dissolved in 20 μL DEPC treating water. Then the concentration of total RNA was detected by using Nano-Drop (Thermo, American). The reverse transcription system had 20 μL materials and after adding in 1.5 μg total RNA, they were removed the genomic DNA by using RT-PCR Kit (Takara) and then were conducted to a reverse transcriptional reaction according to the manual operation steps. The obtained reverse transcripts were diluted 10 times and subsequently conducted to a real-time PCR amplification by using real-time qPCR (ABI 7500, American) and SYBR Green kit (Takara). With the β-actin as a reference, the relative gene expression level was calculated by using $2^{-\Delta\Delta Ct}$ method. The primers were synthesized by Shanghai Sangon Biotechnology Company (Table 1).

2.6. Western blot

The macrophage was processed by using 100 ng/mL of Poly-ICLC for 30 min, and after 1 h, the cells were collected. The precooling PBS, after washing, were conducted to a cell lysis by using lysis buffer contained with 1 M tris–HCl pH 7.5, 1% TritonX-100, 1% NP-40, 10% SDS, 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF, 40 mmol/L DTT, phosphatase inhibitors and protease inhibitor. After centrifugation at 4 °C 12000 r/min for 20 min, the supernatant was collected and then tested by Bradford method. The total protein was boiled for 10 min for the denaturation, and the electrophoretic separation and transmembrane of it were then carried out in the SDS-PAGE gel. The PVDF membrane was blocked in tris buffered saline Tween (TBST) of 5% skimmed milk powder for 2 h and then added in primary antibodies for overnight

Table 1
The sequence of real-time PCR primer.

Gene name	Primer sequences
IL-1β-F	5′-AAGCCTCGTGCTGTCGGACC-3′
IL-1β-R	5′-TGAGGCCCAAGGCCACAGGT-3′
TNFα-F	5′-CATCTTCTCAAAATTCGAGTGACAA -3′
TNFα-R	5′-CCAGCTGCTzCCTCCACTTG-3′
iNOS-F	5′-GTTCTCAGCCCAACAATACAA-3′
iNOS-R	5′-GTGGACGGGTCGATGTCAC-3′
Arg-1-F	5′-TGGCTTGCGAGACGTAGAC-3′
Arg-1-R	5′-GCTCAGGTGAATCGGCCTTTT-3′
YM-1-F	5′-AGAAGGGAGTTTCAAACCTGGT-3′
YM-1-R	5′-CTCTTGCTGATGTGTGTAAGTGA-3′
CD206-F	5′-TTCGGTGGACTGTGGACGAGC A-3′
CD206-R	5′-ATAAGCCACCTGCCACTCCGG T-3′
β-actin-F	CGTGAAAAGACCCAGATCA
β-actin-R	CACAGCCTGGATGGCTACGT

incubation at 4 °C. After the TBST was washed for 5 min for 3 times, the second antibody (horseradish peroxidase labeled goat anti rabbit IgG, 1:1 000) was added in and then was incubated at room temperature for 2 h. When the sufficient rinse of TBST for 10 min for total 3 times was finished, the coloration was carried out by using electrochemiluminescence method.

2.7. Statistical analysis

All the experimental data were processed by using GraphPad Prism software and were showed as Mean ± SE. The analysis of the survival curves was tested by applying the Long-Rank. The Means of two groups were tested by using *t*-test and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Significant inhibition of tumor growth by Poly-ICLC

In the subcutaneous tumor animal models of 3LL and B16F10, the tumor growth ratio of mice in Poly-ICLC treating group significantly decreased and the survival time of mice with brown tumor was significantly longer than that in PBS control group with that the tumor size also had significant difference between two groups after 15-d growth of brown tumor (Table 2), which showed that the Poly-ICLC could inhibit the tumor growth.

3.2. Increase of $CD8^+$ T cell ratio and the decrease of Treg ($CD3^+$)

In order to define the mechanism of Poly-ICLC on inhibiting tumor growth, the infiltration of local tumor T cell was detected by using flow cytometry. The result showed that compared with control group, the ratio of Treg of local tumor in mice with brown tumor in treating group notably decreased among the $CD45^+CD3^+$ cells, while the ratio of $CD8^+$ T cell increased significantly, there were significant difference between them (Table 3).

3.3. Changes of macrophage phenotypes

Meanwhile, the significant difference was also found in the expression of M1 and M2 macrophage phenotypes in local tumor. Compared with the PBS group, the expression of CD206 (M2-type markers) in macrophage ($CD45^+F4/80^+$ cells) in treating group was significantly inhibited, while, the expression of iNOS (M1-type markers) increased markedly (Table 4). The results revealed that Poly-ICLC could improve the tumor immune microenvironment.

Table 2
The effect of Poly-ICLC on tumor growth.

Group		PBS group	Poly-ICLC group
3LL	Tumor size (mm^2)	82.73 ± 6.21	48.91 ± 7.32*
	Survival time (d)	21.21 ± 1.32	28.77 ± 2.19*
B16F10	Tumor size (mm^2)	79.13 ± 8.03	42.10 ± 6.52*
	Survival time (d)	20.62 ± 2.41	32.51 ± 3.81*

Compared with PBS group, $^*P < 0.05$.

3.4. M1 macrophage polarization induced by Poly-ICLC

The Poly-ICLC was used to *in vitro* processed the bone marrow-derived macrophage and after 24 h, the changes of the expression of M1/M2 markers were detected by real-time PCR. As shown in Table 5, the expression of M1 polarization genes *iNOS*, *TNF-α* and *IL-1β* of cells treated with Poly-ICLC increases significantly compared with the control group, while the expression of M2 type markers CD206, YM-1 and Arg-1 decreases notably and there is a concentration dependence.

3.5. Activation of toll-like receptors 3 (TLR3) downstream signaling pathways by Poly-ICLC

In order to further discuss the forming pattern of macrophage M1 polarization induced by Poly-ICLC, the TLR3 downstream signaling pathway was tested and the result showed that the Poly-ICLC could caused the significant increased phosphorylation levels of p65, TBK1 and IRF3 of TL3 downstream signaling pathways in macrophage (Table 6), which further confirm that Poly-ICLC could activate TLR3 downstream signaling pathways in macrophages.

4. Discussion

A large number of animal experiments and clinical test have verified that M2 type TAM can promote the growth, infiltration and migration of tumor, while the M1 type TAM possesses the effect of promoting immune activation and inhibiting tumor growth. Therefore, it may be a new breakthrough point for the future cancer treatment to further explore the transformation between these two cell types, confirm their signal transduction

Table 3
The infiltration of Treg and $CD8^+$ T cells in the local tumor.

Ratio/$CD45^+$(%)	PBS group	Poly-ICLC group
Treg	21.38 ± 4.76	9.14 ± 2.06*
$CD8^+$ T cell	19.95 ± 5.31	38.97 ± 6.55*

Compared with the control group, $^*P < 0.05$.

Table 4
Expression of M1/M2 type markers in the macrophage.

M1/M2 markers (%)	PBS group	Poly-ICLC group
CD206	48.62 ± 9.62	17.57 ± 7.13*
iNOS	2.67 ± 0.48	6.03 ± 2.75*

Compared with the control group, $^*P < 0.05$.

Table 5
M1 macrophage polarization induced by Poly-ICLC.

Gene expression		Control	Poly-ICLC	
			10 ng/mL	100 ng/mL
M1 type	*iNOS*	1.02 ± 0.02	34.67 ± 5.21*	88.04 ± 18.35**
	TNF-α	1.00 ± 0.11	213.16 ± 51.06*	871.81 ± 136.21**
	IL-1β	0.98 ± 0.12	371.39 ± 77.14*	572.81 ± 97.41**
M2 type	*CD206*	1.01 ± 0.13	0.42 ± 0.11*	0.35 ± 0.08*
	Arg-1	1.04 ± 0.07	0.36 ± 0.17*	0.13 ± 0.11*
	Tim-1	0.98 ± 0.05	0.48 ± 0.12*	0.42 ± 0.07*

Compared with the control group, $^*P < 0.05$; $^{**}P < 0.01$.

Table 6
Phosphorylation of p65, TBK1 and IRF3Poly-ICLC promoted by Poly-ICLC.

Protein expression	Control	Poly-ICLC	
		30 min	60 min
p-p65	1.04 ± 0.01	2.37 ± 0.26*	5.42 ± 0.47**
p-TBK1	0.89 ± 0.21	3.60 ± 0.66*	7.81 ± 1.22**
p-IRF3	0.97 ± 0.15	2.33 ± 0.34*	6.03 ± 1.05**

Compared with the control group, $^{*}P < 0.05$; $^{**}P < 0.01$.

pathways and molecular mechanisms, to alter local neoplasm M2 macrophages to M1 macrophages polarization through artificial means.

Pattern recognition receptors (PPR) plays a key role in the mutual recognition and function of pathogen-associated molecular pattern (PAMP) of the host cell surface and pathogen surface, and in the innate immune response activation [6]. Then, the TCR is considered as the most important PPR which can identify a variety of pathogens, activate the natural immune response, remove viruses or exert the antitumor effect [7–9]. TLR3 ligand is a double-stranded RNA which usually does not exist in the mammalian body. It is the product of viral replication, therefore, it is regarded as a crucial 'danger signal' of inducing the body's natural immune response.

The Poly (I:C), a dsRNA analog, consists of a ploy (I) and a ploy (C). The Poly-ICLC is produced by using Poly l lysine and carboxymethylcellulose to stabilize Poly (I:C) and both them are the TLR3 agonists. The Poly-ICLC can induce the Th1 cytokine secretion, such as IL-12, TNF-α, IFN-γ, IL-6 and type I interferon [10–12], and induce the expression of chemokines, such as the expression of CXCL9, CXCL10, MCP1, MIP1-a and MIP1–b. It is closely associated with the immune cells recruitment [13]. The patients with glioma tumor treated by a combination therapy of intramuscular poly-ICLC, radiation and temozolomide, the result showed that Poly-ICLC could improve the effect of radiation and chemotherapy and had no significant toxic and side effect [14]. The Poly-ICLC can also block immunosuppressive molecules signals or other danger signals and then further improve the results of cancer vaccines. It has been used as the immunologic adjuvant in the treatment of lymphoma, liver cancer, colon cancer and leukemia *etc*., which has achieved a certain curative effect [15–17]. The result obtained in this study also showed that the Poly-ICLC could extend the survival time of mice with brown tumor, inhibit the tumor growth and had the antitumor effect.

The TLR3 can express in a variety of tissue cells including epithelial cells, tumor cells and immune cells *etc*. The previous study has reported that Poly-ICLC can induce the tumor cell apoptosis by activating the downstream signaling pathway [18], while the activation of TLR3 in lymphocyte can induce an innate immune response [19]. The result showed that the ratio of Treg in local tumor of mice treated with Poly-ICLC decreased significantly, while, the ratio of $CD8^+$ T cell increased, which could showed that the immune suppressive microenvironment of it had been improved. The Poly-ICLC could caused a increased expression of iNOS (macrophage phenotype M1 markers) in local tumor and inhibit the expression of CD206 (M2 markers), which revealed that Poly-ICLC might have an effect on inducing the M1 polarization of TAM.

Through the *in vitro* cultivation of mouse bone marrow-derived macrophage and after 24-h proceeding by Poly-ICLC, the expression of proinflammatory factor iNOS, TNF-α and IL-1β in macrophage significantly increased, while the expression of inhibitory molecules CD206, YM-1 and Arg-1 decreased notably with that the expression difference was positively correlated with the concentration of Poly-ICLC, which showed that Poly-ICLC could directly affect the macrophage and induce the changes in the state of immune function of macrophage. Besides, the western blot result revealed that 0.5 and 1 h after received the stimulation from Poly-ICLC, the phosphorylation levels of p65, TBK1 and IRF3 of TL3 downstream signaling pathways in macrophage increased significantly, which further proved that Poly-ICLC could activate TLR3 downstream signaling pathway in macrophages to cause the M1 polarization.

In conclusion, in the subcutaneous transplantation tumor models of mice Lewis lung cancer 3LL and C16B10 melanoma cells, Poly-ICLC can be used as a TLR3 agonist to activate the downstream signaling pathway, induce the polarization of M1 TAM, improve the local inhibitory tumor microenvironment and then exert the antitumor effect. Poly-ICLC can effectively activate the innate immune cells, which has a wide application prospect in anti-tumor treatment.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. *Trends Immunol* 2012; **33**(3): 119-126.

[2] Cui R, Shi XY. Expression of pyruvate kinase M2 in human colorectal cancer and its prognostic value. *Int J Clin Exp Pathol* 2015; **8**(9): 11393-11399.

[3] Komura T, Sakai Y, Harada K, Kawaguchi K, Takabatake H, Kitagawa H, et al. Inflammatory features of pancreatic cancer highlighted by monocytes/macrophages and $CD4^+$ T cells with clinical impact. *Cancer Sci* 2015; **106**(6): 672-686.

[4] Zhang BC, Zhang YF, Zhao J, Wang ZG, Wu TT, Ou WL, et al. M2-polarized macrophages contribute to the decreased sensitivity of EGFR-TKIs treatment in patients with advanced lung adenocarcinoma. *Med Oncol* 2014; **31**(8): 127.

[5] Becker M, Müller CB, De Bastiani MA, Klamt F. The prognostic impact of tumor-associated macrophages and intra-tumoral apoptosis in non-small cell lung cancer. *Histol Histopathol* 2014; **29**(1): 21-31.

[6] Sellge G, Kufer TA. PRR-signaling pathways: learning from microbial tactics. *Semin Immunol* 2015; **27**(2): 75-84.

[7] Jiménez-Dalmaroni MJ, Gerswhin ME, Adamopoulos IE. The critical role of toll-like receptors – from microbial recognition to autoimmunity: a comprehensive review. *Autoimmun Rev* 2016; **15**(1): 1-8.

[8] van Egmond M, Vidarsson G, Bakema JE. Cross-talk between pathogen recognizing Toll-like receptors and immunoglobulin Fc receptors in immunity. *Immunol Rev* 2015; **268**(1): 311-327.

[9] Adams S. Toll-like receptor agonists in cancer therapy. *Immunotherapy* 2009; **1**(6): 949-964.

[10] Gesuete R, Packard AE, Vartanian KB, Conrad VK, Stevens SL, Bahjat FR, et al. Poly-ICLC preconditioning protects the blood-brain barrier against ischemic injury *in vitro* through type I interferon signaling. *J neurochem* 2012; **123**(Suppl 2): 75-85.

[11] Salazar AM, Erlich RB, Mark A, Bhardwaj N, Herberman RB. Therapeutic *in situ* autovaccination against solid cancers with intratumoral poly-ICLC: case report, hypothesis, and clinical trial. *Cancer Immunol Res* 2014; **2**(8): 720-724.

[12] Martins KA, Steffens JT, van Tongeren SA, Wells JB, Bergeron AA, Dickson SP, et al. Dickson SP2, Toll-like receptor agonist augments virus-like particle-mediated protection from Ebola virus with transient immune activation. *PLoS One* 2014; **9**(2): e89735.

[13] Zhu X, Fallert-Junecko BA, Fujita M, Ueda R, Kohanbash G, Kastenhuber ER, et al. Poly-ICLC promotes the infiltration of effector T cells into intracranial gliomas via induction of CXCL10 in IFN-alpha and IFN-gamma dependent manners. *Cancer Immunol Immunother* 2010; **59**(9): 1401-1409.

[14] Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M, et al. Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the United States. *Clin Cancer Res* 2010; **16**(8): 2443-2449.

[15] Martins KA, Bavari S, Salazar AM. Vaccine adjuvant uses of poly-IC and derivatives. *Expert Rev Vaccines* 2015; **14**(3): 447-459.

[16] Glavan TM, Pavelic J. The exploitation of Toll-like receptor 3 signaling in cancer therapy. *Curr Pharm Des* 2014; **20**(42): 6555-6564.

[17] Rosenfeld MR, Chamberlain MC, Grossman SA, Peereboom DM, Lesser GJ, Batchelor TT, et al. A multi-institution phasellstudy of poly-ICLC and radiotherapy with concurrent and adjuvant temozolomide in adults with newly diagnosed glioblastoma. *Neuro Oncol* 2010; **12**(10): 1071-1077.

[18] Gambara G, Desideri M, Stoppacciaro A, Padula F, De Cesaris P, Starace D, et al. TLR3 engagement induces IRF-3-dependent apoptosis in androgen-sensitive prostate cancer cells and inhibits tumour growth *in vivo*. *J Cell Mol Med* 2015; **19**(2): 327-339.

[19] Ho V, Lim TS, Lee J, Steinberg J, Szmyd R, Tham M, et al. TLR3 agonist and Sorafenib combinatorial therapy promotes immune activation and controls hepatocellular carcinoma progression. *Oncotarget* 2015; **6**(29): 27252-27266.