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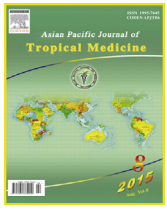

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Correlation between TAMs and proliferation and invasion of type I endometrial carcinoma

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ABSTRACT

Objective: To explore the correlation between tumor-associated macrophages and the proliferation and invasion of type I endometrial carcinoma.
Methods: Immunohistochemistry was used to investigate the infiltration of macrophages in normal and different types of hyperplastic endometrial lesions. The proliferation and invasion ability of type I endometrial carcinoma cell line RL95-2 influenced by mononuclear macrophage cell line THP-1 (constructed M2 type macrophages) was detected by CCK8 and transwell technologies respectively. Transwell was used to evaluate the recruiting ability of RL95-2 on THP-1 cells. Otherwise, the western blot was also used to detect the expression of CyclinD1 and MMP-2 in RL95-2 with the influence of THP-1.
Results: Immunohistochemistry result showed a positive correlation between the number of infiltrating macrophages and the progression of endometrial hyperplasia. THP-1 recruited by RL95-2 could promote its proliferation and invasion and enhance the expression of the CyclinD1 and MMP-2 protein in a time dependent manner ($P < 0.05$).
Conclusions: Increase of the number of infiltrating macrophages and its contribution to the tumor inflammatory microenvironment may result in the development of the type I endometrial carcinoma.

1. Introduction

The results of epidemiological case studies and investigations have shown that, the incidence of endometrial carcinoma has significant correlation between insulin resistance/metabolic syndrome, non steroid hormone, inflammation and other factors. But the current studies on physiopathologic mechanism of relationship between inflammation and endometrial carcinoma and its molecular biology research have not clearly revealed their specific relationship [1,2]. Basic research has confirmed that, in contrast with other common gynecological tumors, inflammatory microenvironment is more obviously one of the important local characteristics in endometrial carcinoma. And for patients, the local uterine inflammation because of menstrual period and chronic inflammation caused by obesity in female patients with obesity and metabolic syndrome indicate endometrial carcinoma is more closely linked to inflammation [3].

Tumor-associated macrophages (TAMs) is a kind of very important cell group in the stroma of endometrial carcinoma. Its effect on the tumor microenvironment also influences the progression of the disease to a certain extent. It has important significance on the formation of tumor microenvironment. And recent studies have shown its relationship with tumor inflammatory environment, tumor proliferation and tumor invasion [4–6]. Classical activated macrophages (M1) and selective activated macrophages (M2) are two major cell subtypes formed by monocytes/macrophages through activation induction. The main difference between M1 type and M2 type macrophages is different expression of cytokines. M1 type macrophages has the characteristic of expressing inflammatory cytokines, chemokines and effector molecules, while on the contrary, M2 type macrophages can express a large number of anti-inflammatory factors [7]. Lots of basic studies have reported that the M2 type macrophages is also called TAMs because it can infiltrate most tumors. It mainly exists in necrotic area, infiltrating edge, epithelium and stroma of tumor, as an important biological marker of tumor and suggesting the progression of tumor [8].

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Lots of clinical studies have suggested that the poor prognosis of endometrial carcinoma has certain correlation with the increase of TAMs. But current studies have not made clear reports on the relationship between TAMs and the progression of endometrial carcinoma and the detailed mechanism [9,10]. In this study, we detected the macrophage distribution in the local lesions in patients with endometrial hyperplasia, thus to explore a series of biological behavior appearing in the development of endometrial carcinoma and its possible mechanism.

2. Materials and methods

2.1. Specimen collection and immunohistochemical staining

Cases of endometrial carcinoma were selected, including proliferative phase and secretory phase in endometrial carcinoma, simple hyperplasia and complex endometrial carcinoma and atypical hyperplasia, type I endometrial carcinoma, with 25 cases for each type. After being embedded in paraffin, the case tissues were continuously sliced into 5 μm sections by slicing machine. And then routine immunohistochemical ABC staining was used: sectioning, dewaxing and hydrating of the tissues and incubation with 3% H_2O_2 at room temperature for 5–10 min; antigen repairing with Tris-EDTA; sealing with 5%–10% normal goat serum (diluted by PBS); incubation with primary antibody CD68 (SANTA, dilution multiple 1:300) and then biotinylated secondary antibody and rinse with PBS, incubation for 2 h with ABC, rinse with PBS, and coloration with DAB; redyeing, dehydration and making sections. The results were observed and analyzed: the macrophages were observed and counted under the light microscope.

2.2. Methods of cell culture

In the experiment, using human monocyte cell line THP-1 and human type I endometrial carcinoma cells RL95-2 (the required cell lines were both experimental cryopreserved cells in the hospital), suspension subculture was performed with 10% fetal bovine serum in the incubator with an environment of 5% CO_2. The changing liquid and subculture way of THP-1 was that changing liquid or subculture was performed immediately after the centrifugation with 800 r/min rotation speed for 3 min, after which the cells were observed under the microscope to ensure that the cells were still in single, translucent and round suspension state.

2.3. Differentiation induction and phenotype identification

The cultured THP-1 monocytes were used to be inoculated with a density of 1×10^6/mL. The way of adherent induction was adding 70 nm/L phorbol ester (Sigma) before setting aside for 30 h. Then when the cells were observed under the microscope to be adherent, IL-4 and IL-13 (Sigma, 20 nmol/L) were added into a super clean bench. After sealing flask, the cells were continued to be placed in incubator for culture of 18 h, thus M2 type macrophages can be obtained through induction. RT-PCR was used for phenotype identification. The THP-1 cells before and after induction were collected and placed in reserve. The total RNA was extracted by routine methods. With 2.5 μg extracted total RNA, cDNA was obtained through reverse transcription before amplification. Amplification conditions were as follows: pre denaturation for 5 min at 95 °C, denaturation for 5 s at 95 °C, annealing for 30 s at 59 °C and extension for 30 s at 72 °C. With a total of 40 amplification cycles, the added primer design sequences were shown in Table 1.

2.4. Preparation of conditional medium

After the differentiation induction and the treated cells were observed to be still and completely adherent, fresh medium could be used for replacement. The medium used for cell culture was collected 24 h later. After the centrifugation with 4000 r/min rotational speed for 10 min, the obtained supernatant was collected. Pay attention not to collecting the liquid of gradient layer. Then the required conditional medium (CM) was obtained. THP-1 and RL95-2 cells were inoculated according to a cell density of 1×10^6/mL and then differentiation induction or adherence was performed, after which CM-V (THP-1) and CM-R (RL95-2) were prepared using the above method and preserved in a −80 °C refrigerator.

2.5. Determination of RL95-2 growth curve with CCK8

The R95-2 cells were inoculated in 96 hole plate with a density of about 2000/hole. After plating, the super clean bench was taken out carefully and placed under the microscope to observe whether the cells were plated evenly. The cross method was used for sloshing when uneven. After the cells were observed to be even, adherent and still, cells in the control group were added into normal medium (NM) for culture and cells in the experimental group were added into CM-T in a

Table 1
RT-PCR primers.

Gene	Sequence (5′-3′)
CD68	F: CTACGCCTCTTATCTCA R:GTTGGTTGCTTGTGCTTGGTA
CD163	F:TTTTGTCACCAGTTCTCTTGGA R:AGCCATTATTACACACGTTCC
CD204	F:TTCACCAGTATGCAGAGACAC R:TCCGAACTGGGATAGAGGAACT
CD206	F:GGATAAGGAACGTGCAGATTAG R:CTTGACTTATGGCGTCTGCAAA
GAPDH	F:AGAGTGCGTAGGGACCATTTG R:GTAGAGCGTTGGGTTGACGT

super clean bench. Four determination time points were set in each group, with 6 holes in each time point. The spectrophotometric values were determined by CCK8 for 4 consecutive days from 24 h after plating. One-way analysis of variance was used for the results and the growth curve was drawn for comparison.

2.6. Methods of co-culture

After activation induction, M2 type macrophages THP-1 became end cells without proliferation ability. After co-culture with RL95-2, the effect of THP-1 on the proliferation of RL95-2 was determined by CCK8. The specific experimental methods were as follows: in the experimental group the cells were inoculated in 96 hole plate in accordance with 4 gradient ratios of 1:1, 1:2, 1:3 and 1:6 (THP-1: RL95-2) (with a total cell number of 1 000 in each hole), while in the control group 1, 2, 3, 6 copies of RL95-2 cells or 1 copy of THP-1 cells were inoculated respectively with 6 holes in each group. The absorbance values (D) were determined by CCK8 at 48 h after inoculation. The difference between the D values of co-culture groups and THP-1 single culture group was compared with the D values of other 4 RL95-2 single culture groups to detect the proliferation effect. The results were analyzed with statistical one-way analysis of variance.

2.7. Transwell methods of invasion and migration experiment

In the invasion experiment, the experimental materials were precooled. After freezing and thawing overnight at 4 °C and a 1:5 dilution, the BD matrigel was plated in the transwell chambers (30 μL/chamber) and then put in 37 °C incubator to make the matrigel completely solidified. In the experiment of RL95-2 recruiting THP-1, the required cells were processed to 2×10^5/mL. In the control group, 200 μL THP-1 cells were added to the upper chambers and only 600 μL RPMI/1640 medium was added to the lower chambers. In the experimental group, 200 μL THP-1 cells were added to the upper chambers and 600 μL RL95-2 cells were added to the lower chambers. In the experiment of M2 type THP-1 cells promoting the proliferation and invasion of RL95-2, 200 μL RL95-2 cells were added to the upper chambers in both experimental and control group, while 600 μL CM-T was added to the lower chambers of experimental group and 600 μL RPMI/1640 medium was added to the lower chambers of control group. After culturing cells for 24 h, the upper chambers were taken out abandoning the liquid, cleaned with PBS for 3 times and soaked in the fixative for 15 min (preparation method of fixative: methanal and glacial acetic acid were mixed with a ratio of 3:1). After staining for 2 h with 0.1% crystal violet (Sigma), the residual stain was washed away and the chambers were washed with clean water for more than 3 times until the bottoms of the chambers were clean. The observation and counting methods were as follows: the inverted chambers were photographed under a light microscope in selected appropriate fields, and the cells were counted in randomly selected high power fields (×400), making sure that the 5 fields were nonoverlapping. The counting results were analyzed using statistical *t* test.

The steps of migration experiment were the same as that of invasion experiment except no matrigel.

2.8. Western bolt experiment

The total protein was extracted with routine methods at 0, 24, 48, 72 h after the RL95-2 cells were cultured in the CM-T medium. The gel electrophoresis experiment was performed at constant voltage (SDS-PAGE, with 30 μg protein sample) until the required bands were certainly separated before transfer membrane (300 mA, 9 0 min). Then the protein sample was blocked for one night with the 5% skim milk as blocking buffer before adding antibodies. The primary antibodies ERα antibody and Cyclin D1 antibody (company: SANTACRUZ, dilution ratio: 1:1 000) were incubated at 4 °C overnight. And the horseradish peroxidase marked secondary antibody was incubated for 1 h before developing avoiding light. The blotting bands were analyzed using analysis software Image J, and the gray values were analyzed using one-way analysis of variance.

2.9. Data processing

The data used in the study were inputted into GRAPH PAD 5.0 analysis software package. Measure data were shown as mean ± standard deviation (mean ± sd). The differences between groups were compared by *t* test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Relationship between number of infiltrating macrophages and progression of endometrial lesions

Through the analysis of immunohistochemical staining result figures (Figure 1), the infiltration of macrophages in different types of endometrial lesions was as follows: in the proliferative phase of normal tissue, there were 9.37 CD68(+)cells/high power field on average, and the few cells showed an uneven and casual distribution mostly around blood vessels; in the secretory phase, there were 12.57 CD68(+)cells/high power field on average with enhanced staining effect; in the simple hyperplasia endometrial tissue, there were 15.49 CD68(+)cells/high power field on average under the microscope with a casual distribution; in the tissue of complex and atypical hyperplasia endometrial carcinoma, there were 25.72 CD68(+)cells/high power field on average, and the macrophages relatively gathered into a mass with obvious phagocytic vacuoles and phagocytic granules, whose distribution showed a tendency to infiltrate from interstitium to glands; in the tissue of type I endometrial carcinoma, there were 35.84 CD68(+)cells/high power field on average, with gathering distribution, obvious phagocytic vacuoles and phagocytic granules under the microscope, and significant infiltration of phagocytes appeared in the cancer nests, central necrotic areas, interstitium and lesion edges of the lesion area.

3.2. Differentiation induction and phenotype identification of human macrophages

After phorbol ester was added into the nutrient solution of human monocyte cell line THP-1, the cells began to stop proliferating and the cell *CD68* expression was proved to increase by experiment, which indicates the successful induction of macrophages; the cells were made to differentiate into M2

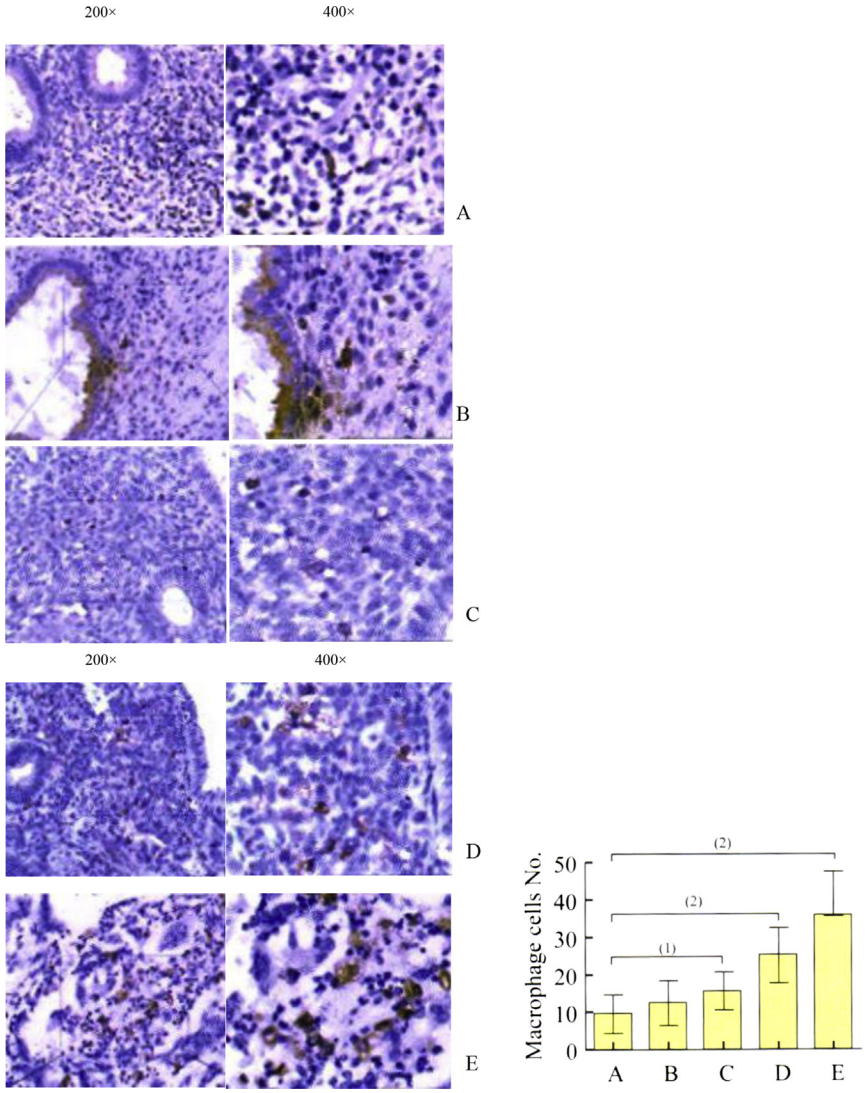

Figure 1. Infiltration of macrophages in different types of endometrial hyperplasia.
A: proliferative phase; B: secretory phase; C: simple hyperplasia; D: complex and atypical hyperplasia; E: type I endometrial carcinoma. (1) $P < 0.01$, (2) $P < 0.001$.

type macrophages through adding IL-4 and IL-13. The cellular morphology before and after activation induction is shown in Figure 2A. The identification results of RT-PCR experiment are shown in Figure 2B. The results show that after differentiation induction, the specific markers including *CD68*, *CD163*, *CD204* (scavenger receptor), *CD206* (mannose receptor) detected by RT-PCR increase significantly, which suggests the formation of M2 type macrophages.

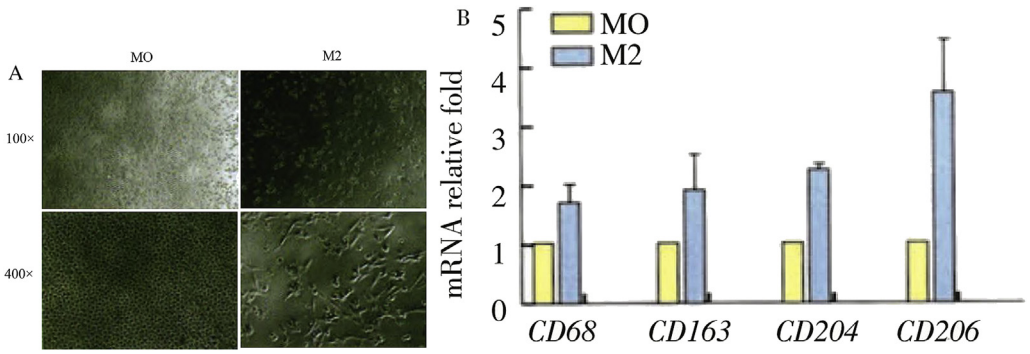

Figure 2. Morphology of THP-1 before and after activation induction and identification results of RT-PCR.
A: THP-1 shows adherence through induction of phorbol ester and activation of IL-4 and IL-13; B: mRNA relative expression of *CD68*, *CD163*, *CD204* and *CD206*. MO: human monocytes; M2: macrophages formed by induction.

3.3. Recruitment and activation induction effect of RL95-2 on THP-1

THP-1 monocytes can be recruited by RL95-2: The results of transwell experiment show that compared with the control group, the THP-1 monocytes (MO) in the upper chambers can invade with the induction of RL95-2 inoculated in the lower chambers, which indicates that RL95-2 cells can recruit THP-1 monocytes. At 24h after THP-1 inoculation, the THP-1 cells were observed to pass through the chambers, which were counted under the microscope. Through statistical analysis of the results, the difference between two groups is statistically significant (Figure 3A, B).

THP-1 monocytes became M2 type macrophages through differentiation induction: after the THP-1 monocytes were cultured in CM-R for 48 h, the total protein was extracted for RT-PCR experiment. The results show that compared with the cells cultured in NM, the M2 type specific markers *CD68*, *CD163*, *CD204* and *CD206* in the cells of experimental group all show increase in transcription level, indicating that in addition to recruiting THP-1 to enter the inflammatory microenvironment, RL95-2 can induce its differentiation into M2 type macrophages (Figure 3C).

3.4. Enhancement effect of M2 type THP-1 on the proliferation ability of RL95-2

M2 type THP-1 could enhance the proliferation ability of RL95-2: The results of CCK8 experiment showed that the proliferation ability of RL95-2 cultured in M2 type conditional medium CM-T was enhanced obviously when compared with that in NM, with a 72 h proliferation rate of 1.104 597 (Figure 4A). In the co-culture condition of RL95-2 and M2 type THP-1 monocytes with 3 different ratios, the proliferation ability of RL95-2 was enhanced obviously. And the enhancement effect of THP-1 on the proliferation of RL95-2 was most obvious when THP-1: RL95-2 is 1:3 with a proliferation rate of 1.479 516 (Figure 4B).

It is shown by western blot experiment that after culture of 1, 34, 48, 72 h in CM-T, the expression of CyclinD1 protein in RL95-2 cells was increased gradually, and it showed a positive correlation with culture time. Compared with the control group, the difference of band gray values was statistically significant ($P < 0.05$). The results indicated that THP-1 enhanced the proliferation ability of RL95-2 by promoting their expression of CyclinD1 (Figure 4C, D).

3.5. Enhancement effect of M2 type THP-1 on the invasion and migration ability of RL95-2

M2 type THP-1 can enhance the invasion and migration ability of RL95-2: The results of transwell experiment show that M2 type THP-1 conditional medium has a significant promoting effect on the invasion and migration ability of RL95-2 cells (Figure 5A). After the plating of matrigel, the RL95-2 cells passing through the upper chambers are less in the control group (9.37 cells/high power field on average in the counting under the microscope). While in the experimental group, the RL95-2 cells passing through the upper chambers increase significantly (24.9 cells/high power field on average in the counting under the microscope) in the condition of adding M2 type CM-T to the lower chambers, and the cells gather in the center of membrane

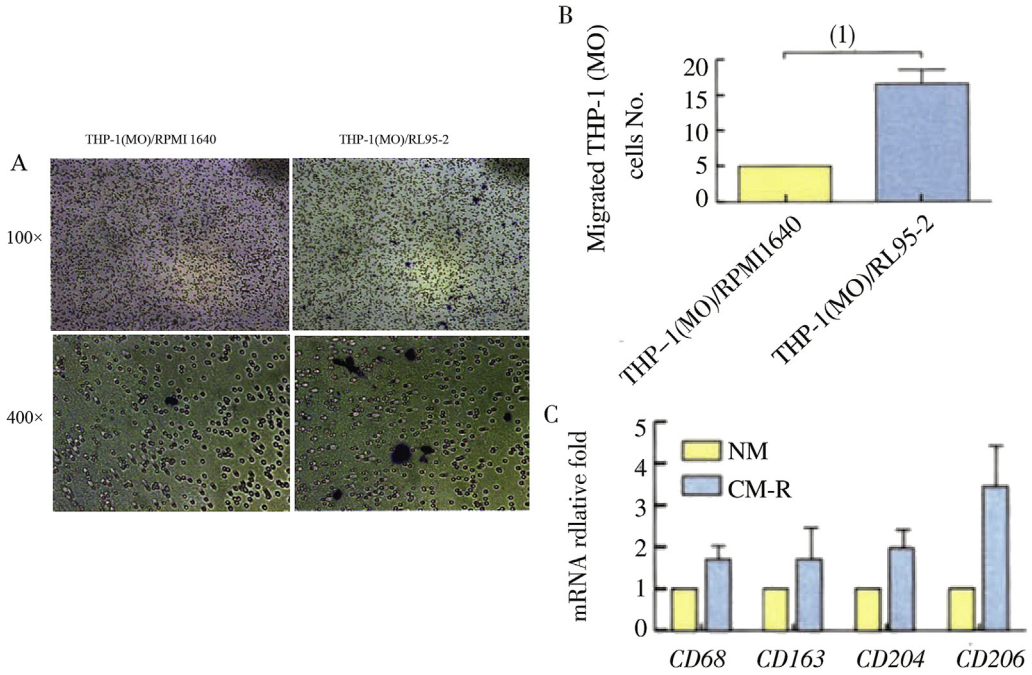

Figure 3. Recruitment and differentiation induction into M2 type macrophages effect of RL95-2 on THP-1.

A: It is shown in the figures under the light microscope that the THP-1 can be recruited by RL95-2. THP-1 (MO)/ RPMI 1640: in the control group, there is NM RPMI 1640 in the lower chambers and THP-1 cells (MO) in the upper chambers; THP-1 (MO)/RL95-2: in the experimental group, there is medium with RL95-2 cells in the lower chambers and THP-1 cells (MO) in the upper chambers.

B: The number of invasive THP-1 cells is counted in five random 400 time's fields without repeat. Compared with the control group, there are more invasive cells in the experimental group.

C: The mRNA relative expression of *CD68*, *CD163*, *CD204* and *CD206* of THP-1 monocytes cultured in NM (NM) and THP-1 monocytes cultured in CM-R (CM-R).

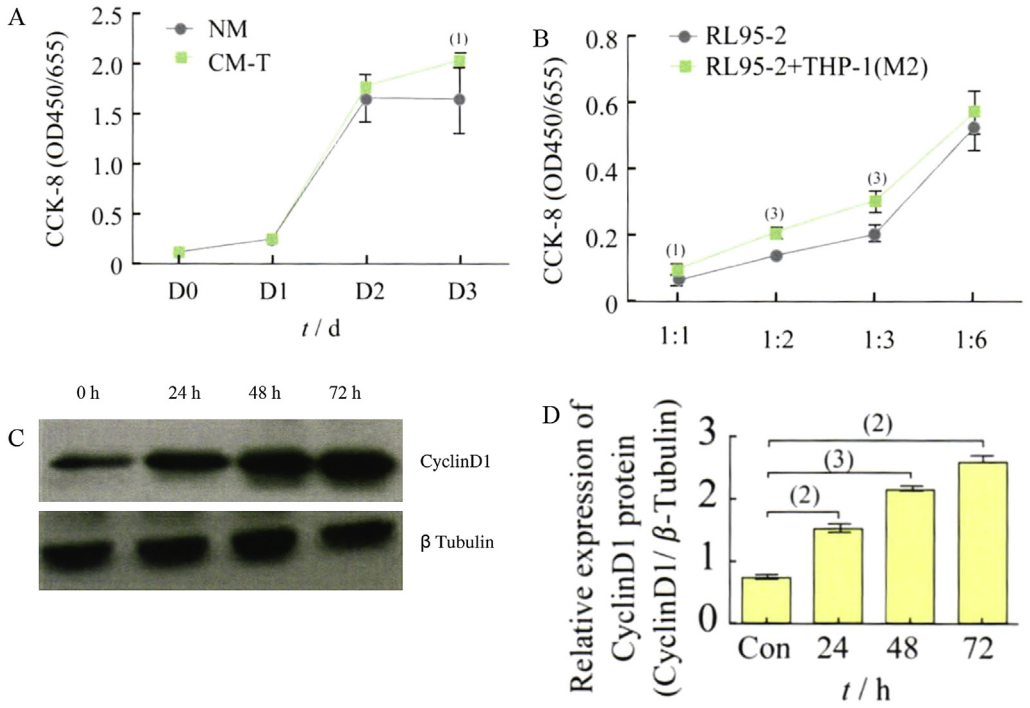

Figure 4. Enhancement effect of THP-1 macrophages (M2 type) on the proliferation ability of RL95-2.
A: Enhancement effect of CM-T on the proliferation ability of RL95-2; B: Enhancement effect in the co-culture of THP-1 and RL95-2 with different ratios; C: Effect of culturing in CM-T for some time on the expression of CyclinD1 protein in RL95-2 cells; D: Relative expression of CyclinD1 protein in RL95-2 cells (CyclinD1 gray value/βTubulin gray value)after culturing in CM-T for some time. (1) $P < 0.05$, (2) $P < 0.01$, (3) $P < 0.001$. NM: normal medium. CM-T: conditional medium of THP-1 macrophages (M2 type).

mostly with a few at the edge of membrane. The results of cell counting show that the difference between the experimental group and the control group has statistical significance ($P < 0.05$) (Figure 5B).

The results of western blot experiment show that after culture of 1, 34, 48, 72 h in CM-T, the expression of MMP-2 protein in RL95-2 cells increases gradually, and it shows a positive correlation with culture time. Compared with the control group, the

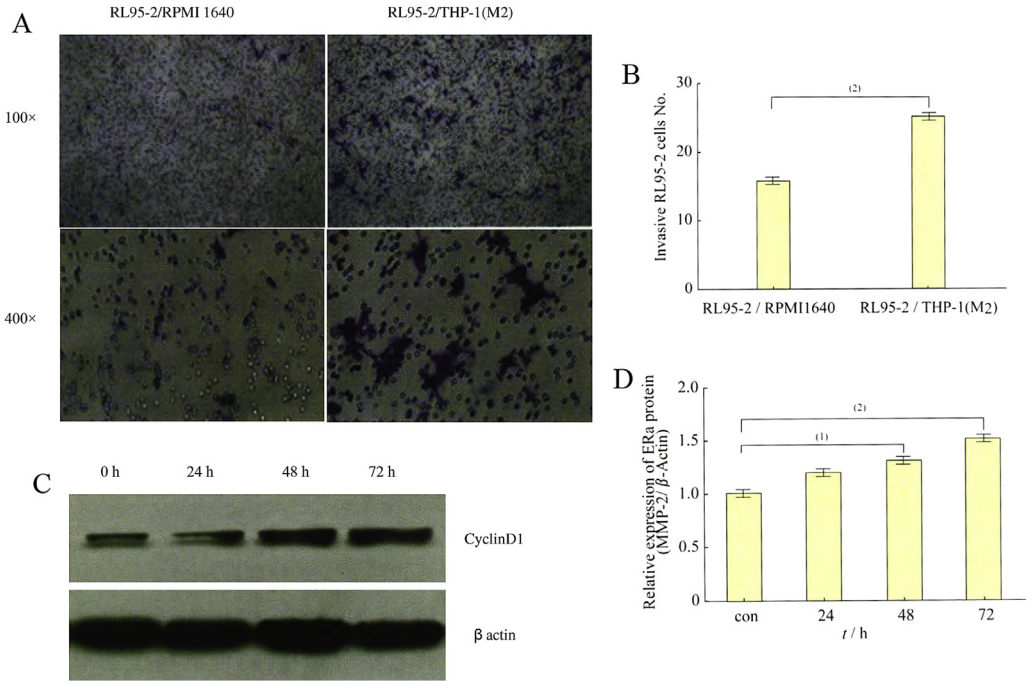

Figure 5. Enhancement effect of THP-1 macrophages (M2 type) on the invasion ability of RL95-2.
A: Enhancement effect of CM-T on the invasion ability of RL95-2. RL95-2/RPMI 1640: there are RL95-2 cells in the upper chambers and the NM RPMI 1640 in the lower chambers; RL95-2/THP-1(M2): there are RL95-2 cells in the upper chambers and THP-1 (M2 type) conditional medium in the lower chambers. B: Comparison of the cell counting results in the chambers of experimental group and control group in five random 400 times fields without repeat under the light microscope. C: Effect of culturing in THP-1 (M2 type) conditional medium for different length of time on the expression of MMP-2 protein in RL95-2 cells; D: Relative expression of MMP-2 protein in RL95-2 cells to that of the control group (0 h) (MMP-2 gray value/βTubulin gray value). (1) $P < 0.05$, (2) $P < 0.01$.

difference of band gray values is statistically significant ($P < 0.05$). The results indicate that THP-1 can enhance the invasion and migration ability of RL95-2 cells by promoting their expression of MMP-2 (Figure 5C, D).

4. Discussion

TAMs is involved in the formation of tumor local inflammatory microenvironment. It has been reported that the infiltration of TAMs has a direct influence on the occurrence and development of malignant tumor [11,12]. A number of important roles played by TAMs have been found in related basic studies on malignant tumor, which mainly include influencing tumor proliferation and invasion, promoting the blood vessel formation effect of tumor in the body local, influencing the signal transduction process in the tumor cells, promoting the development, invasion and drug resistance against cancer drugs of tumor [13–15].

In this study, we explored a series of important effect of TAMs on the development of endometrial carcinoma, the proliferation and invasion of cancer cells and so on. In the infiltration of macrophages, the experiment results show that the number of CD68 (+) cells and the progression of endometrial carcinoma are positively correlated, suggesting that macrophage infiltration and the resulting worse tumor inflammatory microenvironment may played important roles in the progression of endometrial hyperplasia. The morphology of infiltrating macrophages was observed under the microscope. The macrophage morphology is irregular, with large cell body, cytoplasm filled with lots of phagocytic granules and characteristic crescent and horseshoe shaped nucleus. A large number of lymphocytes gather around the macrophages. The morphological characteristics prove that macrophages in the infiltration area of lesions appear in the form of activated and functional cells. It has been reported in other literatures that the distribution of macrophages in the lesions has obvious correlation with the malignant degree and the prognosis of tumor: more and more macrophage infiltration in the cancer nest suggest lower malignant degree of tumor and better prognosis of patient; while a lot of macrophage infiltration detected in the necrotic area suggests higher malignant degree of tumor and poorer prognosis of patient.

In the study, experiments were carried out to study the changes of invasion ability, activation degree and others in THP-1 (having differentiated into M2 type macrophages) with the effect of RL95-2. The results show that the promoting invasion effect of RL95-2 cells is unstable on the THP-1 having differentiated into M2 type macrophages, while the invasion of THP-1 monocytes is promoted obviously in the induction of RL95-2. In addition, the THP-1 cells cultured in the collected CM-R can be induced to differentiate into M2 type macrophages from monocytes. The results suggest that in the endometrial carcinoma tissue of body, cancer cells may make THP-1 cells get involved in the formation of tumor microenvironment by recruiting them and promoting their differentiation into M2 type macrophages, playing an important role in the development of tumor, which is consistent with existing related study results [16,17].

TAMs has very important influence on the proliferation, invasion and migration of endometrial carcinoma cells. It is found in the experiment that with the presence of THP-1 macrophages (M2 type), the expression of CyclinD1 and MMP-2 protein in RL95-2 cells increased significantly and their proliferation, invasion and migration ability are enhanced to some extent. The promoting effect of TAMs on the proliferation and invasion of cancer cells has been reported in other literatures. The characteristic of M2 type macrophages is considered one of the major differences with M1 type macrophages, as well as an important way of the cancer cells to realize immunosuppression [18].

The experimental results show that TAMs and type I endometrial carcinoma seem to have an interaction mode: TAMs participated in the formation of tumor inflammatory microenvironment under the effect of recruitment and differentiation induction of cancer cells; and the recruited TAMs promote the proliferation, invasion and migration of endometrial carcinoma cells, 'assisting' their immunosuppression. Such a mode forms a vicious circle, and ultimately promotes the proliferation and invasion of cancer cells and the tumor to form local inflammatory microenvironment. Such results are also reported in the studies of other cancers, and existing studies suggest that in this process, IL-4 may be an important bridge in mediating the promoting effect of TAMs on the proliferation and invasion of tumor [19,20].

We can draw a conclusion from the above results that in the progression of endometrial carcinoma, the number of infiltrating macrophages has a positive correlation with abnormal and malignant degree of endometrial hyperplasia, and plays an important role in the development of this kind of cancer. Additionally, endometrial carcinoma cells can recruit macrophages and induce their activation to form TAMs, while TAMs can promote the proliferation and invasion of cancer cells by enhancing the expression of CyclinD1 and MMP-2 protein in endometrial carcinoma cells. The 'mutual education' mode between them plays a very important role in the progression of endometrial carcinoma.

Conflict of interest statement

We declare that we have no conflict of interest.

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