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Specific cellular immune response elicited by the necrotic tumor cell-stimulated macrophages

Xiaolin Yuan [a,*], Weina Li [b], Yifen Cui [a], Qing Zhan [a], Chunlei Zhang [a], Zhen Yang [a], Xiaohuan Li [a], Shengfan Li [a], Qinglin Guan [a], Xiuyan Sun [a]

[a] Affiliated Hospital of Dalian University, Dalian 116001, PR China
[b] The Second Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine, Harbin 150001, PR China

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ABSTRACT

Objective: To determine whether the necrotic tumor cell-stimulated macrophages (NTCSM) could elicit specific immune response.
Methods: Mice were immunized with the necrotic H22 tumor cell lysate-stimulated macrophages and the specific immune responses against the same tumor challenge were examined. The morphologic characteristics were observed with the transmission electron microscope and scanning electron microscopy. The expression of CD14, CD68, CD80 and CD86 were detected with the flow cytometer. The cytotoxicity and cytokine production of splenocytes were measured with the MTT assay and ELISA assay respectively.
Results: Our research results reveal that NTCSMs are larger cells which generally generate spherical and elongated protrusions, folding membrane, and vesicles on their surface. Also, abundant lysosomes, secondary lysosomes, phagosomes, rough endoplasmic reticulum, and lipid bodies were found in their cytoplasm. The flow cytometry results show that the necrotic H22 tumor cell lysate could enhance the expression of CD14 and CD86 molecules and the NTCSM was characterized by the expression of CD14+/− CD68 + CD80–CD86+. After the mice were vaccinated with NTCSMs, the tumor forming rate, tumor volume and weight of the NTCSM-vaccinated group were significantly lower than those of the sterile saline-injected group and untreated macrophage-vaccinated group ($p < 0.05$). The cytotoxicity to H22 tumor cells of the splenocytes obtained from the NTCSM-immunized group was higher than that of the sterile saline-injected group and untreated macrophage-vaccinated group ($p < 0.05$). Meanwhile, the levels of IL-2 and IFN-γ in the culture supernatant of the NTCSM-immunized group were higher significantly than those of the saline-injected group and untreated macrophage-vaccinated group. The level of IL-4 of the NTCSM-immunized group was significantly lower than those of the other two groups.
Conclusion: Our results indicated that NTCSMs could elicit specific cellular immune responses in vivo.

1. Introduction

Adjuvants are essential for generation of immunity to many antigens [1]. It has been proven that adjuvant effects occur in the cytoplasm of mammalian cells and the components released from the injured or dead cells can promote the elicitation of cytotoxic T lymphocyte (cytotoxic T lymphocyte, CTL) responses to antigens. CTL responses were increased obviously when the corresponding antigens were co-injected with dying cells [2,3]. However, the mechanism has not been completely understood. Galluchi et al. found that dying cells could stimulate dendritic cells (dendritic cell, DC) to mature and upregulate the expression of co-stimulatory molecules in vitro [4]. Shi et al. found that dying cells could stimulate DCs to acquire antigens, mature, and migrate to lymph nodes in vivo [3]. At present, it is widely accepted that the mechanism of the endogenous adjuvant accounts for the effect of dying cells on DCs. However, we noticed that the necrotic cells could induce CD14–CD68 + macrophages to proliferate and differentiate, and also the differentiated cells possess the biological properties similar to those of macrophages [5]. Macrophages play a key role in the innate and adaptive immune systems respectively. They are multifunctional cells and involved in removing debris, promoting inflammation, generating antigens, and remodeling tissue [6,7]. Macrophage accumulation in situ is a prominent feature for many diseases. The effect of dying cells on macrophages might be another mechanism of the adjuvant function of dying cells.

In this report, it was studied whether the macrophages stimulated by necrotic tumor cell lysate can elicit specific immune responses. The results revealed that immunization with the macrophages induced by necrotic tumor cells significantly reduces the tumor forming and development rate. Our results indicate that macrophages stimulated by necrotic tumor cells could elicit immune responses in vivo.

* Corresponding author at: Research center, Affiliated Hospital of Dalian University, Dalian 116001, PR China. Tel./fax: +86 411 62893508.
E-mail address: xiaolinyuandoc@yeah.net (X. Yuan).

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Table 1
The expression of CD14, CD68, CD80 and CD86 on NTCSM.

	Percentages (mean ± SD, n = 3)			
	$CD68^+$	$CD14^+$	$CD80^+$	$CD86^+$
Non-stimulated macrophages	83.35% ± 9.51%	2.14% ± 0.75%	2.45% ± 0.98%	3.65% ± 1.24%
NTCSM	91.03% ± 8.23%	53.02% ± 9.56%※※	27.53% ± 10.25%※※	84.30% ± 12.71%※※

※※ $p < 0.01$; and ※ as compared with non-stimulated macrophages.

2. Materials and methods

2.1. Mice and cell lines

The Balb/c mice were obtained from Dalian Medical University. The mouse hepatocellular carcinoma cell line H22 was obtained from Harbin Medical University.

2.2. Cytokines and mAbs

The goat anti-mouse CD68, FITC-labeled rabbit anti-goat IgG mAb and isotype-matched antibodies were purchased from Santa Cruz (Santa Cruz, USA). The mAbs, PE-labeled anti-mouse CD14 and CD86, FITC-labeled anti-mouse CD80 and isotype-matched antibodies were ordered from eBioscience (eBioscience, USA). The IL-2, IL-4, IL-10 and IFN-γ ELISA kits were purchased from Dakewe (Dakewe, USA). The Fetal Bovine Serum (FBS) was bought from Sijiqing Company (Sijiqing, China). The RPMI-1640 culture medium was ordered from Gibco (Gibco, USA).

2.3. Preparation of peritoneal macrophages

5 ml of sterile atolin was injected into the peritoneal cavity of the Balb/c mice. 24 h later, all mice were killed by cervical dislocation. Their peritoneal exudate cells were collected by making an incision deep into the peritoneal cavity and the cavity was washed with sterile PBS. The peritoneal exudate cells were cleaned with RPMI 1640 three times and then centrifuged at 800 rpm at 4 °C for 8 min. The cells were allowed to adhere to the substrate for the purpose of culturing them at 37 °C for 24 h. The nonadherent cells were removed by washing with warm PBS three times. At this time, such peritoneal exudate cells should be greater than 90% macrophages.

2.4. Preparation of necrotic H22 cells

H22 cells were adjusted to 2×10^6 cells/ml in RPMI 1640 complete media (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS). The cell suspension was incubated under 5% CO_2 condition at 37 °C for 3 weeks, without changing media or subculturing. After that, the cells were disrupted by four cycles of freezing/thawing followed by sonication.

2.5. Preparation of macrophages stimulated by necrotic cells

The peritoneal macrophages (obtained using the same method as Section 2.3) were treated with 20% necrotic cell lysate under 5% CO_2 condition at 37 °C for 4 days. The dead cell debris was removed by washing the culture dish with PBS three times. The macrophages were digested with 0.25% trypsin-EDTA and then washed with PBS three times.

2.6. Transmission electron microscopy

The cells were fixed in 2% phosphate-buffered glutaraldehyde for 2 h, followed by a post-fixation with 1% phosphate-buffered osmium tetroxide for 1 h. The specimens were dehydrated in the graded solutions of ethanol, dealcoholized with propylene oxide, and embedded in epon 812. Ultrathin sections were double stained using uranyl acetate and lead citrate, and then observed with a transmission electron microscope.

2.7. Scanning electron microscopy

After fixation, the specimens were dehydrated through the graded solutions of ethanol. The following specimens were critical-point dried, mounted on aluminum studs, coated with a 2 nm layer of gold–palladium and examined using a scanning electron microscope.

2.8. Flow cytometry

The cell suspension was washed with ice-cold buffer (PBS containing 0.1% BSA and 0.01% NaN_3) and incubated with FITC or PE-labeled anti-mouse CD14, CD80 and CD86 mAbs for 20 min. For the purpose of detecting CD68 expression, the cells were re-suspended in the ice-cold staining buffer, incubated with the primary mAb at 4 °C for 30 min, washed twice, and then incubated with the secondary FITC-conjugated mAb at 4 °C for 30 min. In this case, the ice-cold buffer and mAb dilution buffer contained 0.5% Triton X-100. After the final washing, the cells were fixed and analyzed using a flow cytometer (Coulter^R, USA).

2.9. Vaccination and tumor challenge

Thirty BALB/c mice were randomly divided into three groups, 10 animals (including 5 males and 5 females) each group, which were vaccinated with a subcutaneous injection of 0.1 ml sterile saline (control group), untreated peritoneal macrophages and necrotic H22 cell lysate-stimulated macrophages in the left hind groin (2×10^5 cells per mouse) on days 14 and 7, respectively. On day 0, the mice were challenged with H22 cells (2×10^7 cells per mouse) by subcutaneous injection in their left fore groin. Those mice were euthanized on day 28 after injection of the H22 cells (Fig. 1).

Table 2
The cytokines production of splenocytes when re-stimulated with H22 cells.

Groups	Levels (pg/ml) (mean ± SD, n = 10)			
	IL-2	IL-4	IL-10	IFNγ
Saline-injected group	2.68 ± 0.28	2.35 ± 0.85	3.03 ± 0.62	4.97 ± 0.72
Untreated macrophage-vaccinated group	3.21 ± 1.23	2.14 ± 0.46	2.96 ± 0.52	5.16 ± 0.89
NTCSM-vaccinated group	8.26 ± 2.36 ※※ * *	0.27 ± 0.14 ※※ * *	2.25 ± 0.37	9.32 ± 1.82 ※※ * *

※※ and **$p < 0.01$; and as compared with saline-injected group, and *as compared with untreated macrophage-vaccinated group.