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Anti-tumor immune responses of tumor-associated macrophages via toll-like receptor 4 triggered by cationic polymers

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ABSTRACT

Agonists of toll-like receptors (TLRs) are potential therapeutic reagents for cancer immunotherapy. Cationic polymers such as polyethyleneimine (PEI) with nucleic acid drug delivery capability are approved for use in clinical trials, and recent reports indicate that these cationic polymers have significant immunological activity mediated by TLRs. In the present study, we demonstrated that cationic polymers such as PEI and cationic dextran could reverse tumor-associated macrophages (TAMs) polarization and promote IL-12 expression both in vitro and in vivo. The stimulatory role of cationic polymers was remarkably attenuated in TAMs pre-treated with TLR-4 blocking antibody or TAMs from TLR-4 knockout mice. Additionally, these cationic polymers exerted direct tumoricidal activity by promoting Th 1 and NK cell infiltration, suppressing tumor angiogenesis and prolonging the survival of sarcoma-bearing wild-type. These phenomena were abrogated in TLR-4 knockout mice, suggesting that the immune stimulation was primarily mediated by TLR-4. In conclusion, these results demonstrated that cationic polymers could transform the immunotolerogenic phenotype of TAMs through TLR-4 signaling, thereby promoting therapeutic anti-tumor immunity. Our present study suggests a new class of drugs as a candidate for future cancer immunotherapy.

1. Introduction

Biomaterials have been widely and successfully used in tissue engineering, the controlled released system of drug delivery, medical implants and biosensors [1]. As foreign to the host, these implanted materials may initiate immunological reactions, and recent studies have revealed some possible mechanisms underlying these immune responses [2–4]. However, there are few studies that report the use of these biomaterials to trigger desired immune responses and to exert specific biological function under certain pathological conditions. Cationic polymers are a class of large molecules that contain repeating structural units rich with amino groups. After being dissolved in water, the amino group becomes protonated and confers a positive charge to the polymer. This cationic polymer is widely used in gene delivery due to its favorable interaction with negatively charged nucleic acid drugs [5]. In our previous in vitro and in vivo studies, a group of cationic polymers such as polyethyleneimine (PEI) and cationic dextran (C-dextran) specifically stimulated macrophages to secret IL-12 production via toll-like receptor 4 (TLR-4) signaling [6]. However, the above research results were carried out in healthy mice with cationic polymer treatment; therefore, its efficacy under certain pathologic states is still undetermined.

Despite great effort to develop various therapeutic and prophylactic treatments, cancer is still the leading cause of death in western countries and the second leading cause of death in developing countries [7]. Cancer immunotherapy attempts to manipulate the exquisite power and specificity of the immune system against tumors and has become a component of some standard cancer treatment regimens. By immune stimulation with cytokines, adjuvants and monoclonal antibodies, immune populations such as NK cells, NKT cells, macrophages and B cells have yielded marked anti-tumor activity [8]. Tumor-associated macrophages (TAMs) are a major component of the leukocytic infiltrate in tumors and have been shown to drive the initiation, proliferation, metastasis and angiogenesis of various tumors [9]. It

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has been demonstrated in various tumors that TAMs represent a major TLR-expressing cell population, and recent studies have indicated that some TLR agonists such as CpG oligonucleotides or M13 bacteriophage could promptly upregulate IL-12 expression and reprogram immunosuppressive TAMs to an immunostimulatory phenotype [10–12]. Based on these findings, we hypothesize that cationic polymers could promote IL-12 secretion in TAMs via TLR-4 signaling and thereby enhance the tumoricidal capacity of TAMs. The present study investigated the possible immunostimulatory effect of these cationic polymers, their mechanism of action on TAMs and their therapeutic potential in a murine allograft tumor model.

2. Materials and methods

2.1. Reagents and C-dextran preparation

PEI (25 kDa), dextran (70 kDa) and 1,1-carbonyldiimidazole (CDI) were purchased from Sigma (St. Louis, MO, USA). Other chemical reagents were purchased from Sangon Biotech (Shanghai, China). C-dextran (9.18% modified) was prepared by incorporating ethylenediamine within the hydroxyl groups of agarose/phytagel by a CDI activation method (mole ratio: dextran: CDI = 1:3), as previously reported [13]. The cationic degrees were determined by element analysis. All synthetic polymers were pre-treated by endoxin removal kit and the content of enodoxin in cationic polymer solution (50 μg cationic polymer/ml) which was determined by tachypleus amebocyte lysate, was lower than 5 EU/ml.

2.2. Animal and tumor model establishment

Male C57BL/10J mice (6–8 weeks old) were purchased from the Experimental Animal Center of Nanjing Medical School (Nanjing, China). C57BL/10ScNJ (TLR-4 KO) mice on the C57BL/10J background were bred in-house using breeding pairs originally purchased from The Model Animal Research Center of Nanjing University (Nanjing, China). All animals were maintained in specific pathogen-free conditions and were treated in accordance with the local policy for animal experiments.

Mouse sarcoma cell line S180 was obtained from ATCC (ATCC® TIB-66™). Cells were grown and maintained in RPMI-1640 medium supplemented with 10% FBS. To generate the heterotopic tumor model, 1×10^6 cells were injected subcutaneously into the left armpit of the animals. Tumor sizes were measured by caliper, and the tumor weights were determined at the time of harvest.

2.3. TAM isolation and treatment

As previously described [14], TAMs were isolated from the tumors one week post-implant in both wide type (WT) and TLR-4 KO mice, and they were subsequently cultured in RPMI-1640 medium containing 10% FBS. Greater than 95% purity of macrophage isolates was determined by immunofluorescent staining for F4/80. Trypan blue staining demonstrated that the viability of isolated TAMs was greater than 90%.

To evaluate the immunologic effect of cationic material, TAMs were incubated with dextran, C-dextran or PEI (50 μg/ml) for 6 h. In blocking assays, cells were pre-incubated for 45 min with 20 μg/ml mouse TLR-4/MD-2 neutralizing antibody (MTS 510, eBioscience), isotype control antibody or PBS prior to incubation with cationic polymers. After treatments, the cells were collected for mRNA quantification (nitric oxide synthase 2 (Nos2); major histocompatibility complex, class II invariant chain (MHCII); arginase 1 (Arg1); and chitinase 3-like 3 (Ym1)) and analysis of secreted cytokines (IL-10 and IL-12p70) by ELISA assays (R&D).

2.4. Anti-cancer activity of cationic polymers

Animals bearing implanted tumors diameter >0.5 cm were administered intratumoral dextran, PEI, C-dextran or methotrexate (MTX) at the dose of 1 mg/kg body weight every two days. Following drug administration, the tumor diameters were examined every two days. All tumors were excised, weighed and sectioned for histopathologic and immunofluorescent analysis on day 14 post-first time treatment with cationic polymers. TAMs isolated from these tumors were used for identification of macrophage markers by real-time qPCR and analysis of secreted cytokines (IL-10 and IL-12). To assess overall survival, tumor-bearing mice were given therapeutic agents intratumorally every 2 days after achieving a tumor burden greater than 0.5 cm. Once the diameter of a tumor reached 2.5 cm, the survival study was terminated, and the mice were humanely sacrificed.

2.5. mRNA quantification

Total RNA from TAMs was prepared using TRIzol reagent (Invitrogen). Real-time qPCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol. Primers for β-actin were used as internal controls. Nos2 sense: 5′-CCAAGCCCTCACCTACTTCC-3′; Nos2 antisense: 5′-CTCTGAGGGCTGACACAAGG-3′; MHCII sense: 5′- CATCTGCTCACGAGGTCTGGA-3′; MHCII antisense: 5′- TGGCACTGGAGTGGCAAATAG-3′; Arg1 sense: 5′-CTCCAAGC-CAAAGTCCTTAGAG-3′; Arg1 antisense: 5′-AGGAGCTGTCATTAGGGACATC-3′; Ym1 sense: 5′-AGAAGGGAGTTTCAAACCTGGT-3′; Ym1 antisense: 5′-GTCTTGCTCATGTGTG-TAAGTGA-3′; β-actin sense: 5′-GGTGTGATGGTGGGAATGGG-3′; β-actin antisense: 5′-ACGGTTGGCCTTAGGGTTCAG-3′.

2.6. Immunofluorescent staining

Frozen tumor sections were incubated with the following primary antibodies: rabbit anti-mouse IFN-γ, rabbit anti-mouse IL-10, rabbit anti-mouse IL-12, rabbit anti-mouse vascular endothelial growth factor (VEGF), rabbit anti-mouse matrix metallopeptidase 9 (MMP9, Boster), rabbit anti-mouse CD31 (Abcam), rat anti-mouse CD4, rat anti-mouse CD49b, or rat anti-mouse F4/80 (eBioscience). Sections were then incubated with the following secondary antibodies: FITC-labeled goat anti-rat, TRITC-labeled goat anti-rat or TRITC-labeled goat anti-rabbit (KPL). All images were captured on a Nikon fluorescent microscope equipped with a digital camera (TE2000-U, Nikon), and images were analyzed using Nis-element Basic research (Nikon) software. Each immunofluorescent stain was repeated three times using serial sections; negative controls were included to determine the amount of background staining.

2.7. Statistics

Results are expressed as the mean ± standard error of the mean (S.E.M). The differences between groups were analyzed by the Mann–Whitney *U* test and, if appropriate, by the Kruskal–Wallis ANOVA test. A value of $p \leq 0.05$ was considered significant. Survival curves were analyzed by the Kaplan–Meyer log-rank test.

3. Results

3.1. The in vitro stimulating effect of cationic polymers on TAMs

After incubation with C-dextran, PEI or unmodified dextran, the supernatant from cultured TAMs was assayed for IL-12 and IL-10. As shown in Fig. 1A, IL-12 was upregulated by the addition of C-dextran or PEI; in contrast, the expression of IL-10 was suppressed by these cationic polymers. As TAMs usually have an IL-10^{high}, IL-12^{low} M2-like phenotype, we examined whether these cationic polymers reversed their phenotype. As demonstrated in Fig. 1B, the TAMs increased their expression of pro-inflammatory genes (NOS2 and MHCII) and reduced their expression of M2-specific genes (Arg1 and Ym1) after cationic polymer stimulation. Our previous studies have shown that cationic polymers induced IL-12 secretion via TLR-4 signaling. Therefore, we used both antibody blocking assays and TLR-4 KO mice to investigate whether TLR-4 signaling by cationic polymers mediates a TAMs phenotype switch. In blocking assays, MTS 510 (TLR-4 blocking antibody) reversed the effect of cationic polymers on the secretion of IL-10 and IL-12 and TAMs phenotype redirection (Fig. 1C, D). TAMs from TLR-4 KO mice failed to respond to stimulation by cationic polymers, as no significant change of IL-10, IL-12 or related macrophage marker expression was observed (Fig. 1E, F).

3.2. The anti-tumor activity of cationic polymers

As TAMs are critical regulators during tumor development, the anti-tumor activity of cationic polymers was evaluated in an allograft model using a sarcoma cell line, S180. Intratumoral injection of C-dextran, PEI and MTX caused a decrease in tumor weight and size (Fig. 2A, B). Overall survival of the mice is shown in Fig. 2C. Tumor-bearing animals administered a saline control died within 4 weeks; in contrast, 50% of mice treated with C-dextran and PEI survived for more than 5 weeks. Fig. 2D shows the image of tumors harvested after two weeks of different treatments. Histologic analysis of tumor sections (Fig. 2E) revealed a large necrotic area of tumors treated with C-dextran, PEI and MTX. C-dextran and PEI exhibited nearly the same efficacy as MTX in preventing tumors.