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Anti-arthritis activity of cationic materials

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Abstract

Cationic materials exhibit remarkable anti-inflammatory activity in experimental arthritis models. Our aim was to confirm this character of cationic materials and investigate its possible mechanism. Adjuvant-induced arthritis (AIA) models were used to test cationic materials for their anti-inflammatory activity. Cationic dextran (C-dextran) with different cationic degrees was used to investigate the influence of the cationic elements of materials on their anti-inflammatory ability. Peritoneal macrophages and spleen cells were used to test the expression of cytokines stimulated by cationic materials. Interferon (IFN)-γ receptor-deficient mice and macrophage-depleted rats were used to examine the possible mechanisms of the anti-inflammatory activity of cationic materials. In AIA models, different cationic materials shared similar anti-inflammatory characters. The anti-inflammatory activity of C-dextran increased with as the cationic degree increased. Cationic materials stimulated interleukin (IL)-12 expression in peritoneal macrophages, and strong stimulation of IFN-γ secretion was subsequently observed in spleen cells. *In vivo* experiments revealed that circulating IL-12 and IFN-γ were enhanced by the cationic materials. Using IFN-γ receptor knockout mice and macrophage-depleted rats, we found that IFN-γ and macrophages played key roles in the anti-inflammatory activity of the materials towards cells. We also found that neutrophil infiltration at inflammatory sites was reduced when AIA animals were treated with C-dextran. We propose that cationic signals act through an unknown receptor on macrophages to induce IL-12 secretion, and that IL-12 promotes the expression of IFN-γ by natural killer cells (or T cells). The resulting elevated systemic levels of IFN-γ inhibit arthritis development by preventing neutrophil recruitment to inflammatory sites.

Keywords: cationic material • arthritis • IL-12 • IFN-γ

Introduction

Rheumatoid arthritis (RA) is a serious health problem that affects about 1% of the world's population. It is a disease that involves complex immunologic mechanisms such as inflammation, immunologic tolerance and autoimmunity. The current therapies for RA include a range of options [1]. Methotrexate (MTX) and other disease-modifying antirheumatic drugs exert their effects through general immunosuppressive properties. More recently, recognition of the importance of cytokine signalling in RA has led to the discovery of cytokine-targeting agents, and such agents are now widely prescribed [2]. Drug discovery strategies targeting T cells and B cells have also proved successful [3]. However, despite this range of options, there remains a substantial unmet medical need in the treatment of RA. For example, the disease fails to respond to anti-tumour necrosis factor (TNF) therapies in a significant percentage of patients, and disease progression frequently occurs in patients whose RA initially responds to therapy [4].

New therapeutic strategies that are more efficient and cost effective are still being urgently explored. The discoveries of alternative drug targets and corresponding medications are the most important research subjects in this field and have attracted more and more researchers and investigations. Over the past few decades, the involvement of interferon (IFN)-γ in the pathology of RA has been profoundly investigated, and deficiency of IFN-γ has been found to exacerbate experimental arthritis in many animal models [5–7]. *In vivo* observations revealed that IFN-γ can reduce the infiltration of neutrophils at inflammatory sites [8, 9]. Another study suggested that IFN-γ suppresses the production of interleukin (IL)-8, which is a specific chemokine for neutrophils [9]. Together with IL-12, IFN-γ can induce cross-talk between $CD8\alpha^+$ dendritic cells and natural killer (NK) cells, which results in suppression of neutrophil recruitment to joints [8]. More importantly, intra-articular injection of recombinant IFN-γ in experimental

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arthritis models ameliorates the symptoms of inflammation [10]. However, clinical trials using IFN-γ as a therapeutic medication for treating RA patients did not produce definite results that this cytokine has therapeutic effects compared with placebos [11]. However, these findings do not mean that IFN-γ cannot be a therapeutic target in the treatment of RA. CpG oligonucleotides that strongly stimulate the production of IFN-γ *in vivo* were found to elicit effective relief of experimental arthritis [8]. These results suggest that agents with the property of stimulating IFN-γ secretion *in vivo* may have potential as therapeutic agents for RA.

The discovery that led to our present study was made by chance. In previous studies involving the treatment of RA experimental models with nucleic drugs delivered by cationic materials, we found that most of the cationic materials used had some ability to ameliorate the symptoms of adjuvant-induced arthritis (AIA) models. In the present study, systematic experiments were performed to clarify these observations. The results demonstrated that relief of inflammation is a universal property of cationic materials including cationic polysaccharides, cationic proteins and polymers such as polyethylenimine (PEI). Such effects became stronger with as the cationic degree of the materials increased. In our experiments, cationic dextran (C-dextran) and PEI had much better therapeutic effects on RA than MTX. Additional investigations suggested that this property of cationic materials was due to their ability to induce the production of IFN-γ *in vivo* and *in vitro*. C-dextran and PEI were able to enhance the secretion of IFN-γ by splenic cells by hundreds of times. Further experiments revealed that macrophage-secreted IL-12 induced by cationic materials may be the origin of the whole signal cascade. Taken together, these findings suggest that cationic materials, especially PEI and C-dextran, have the potential to be therapeutic agents for RA treatment. Most importantly, this novel strategy may lead to new investigations into drug discovery and pathology exploration for RA.

Materials and methods

Cationic materials

PEI, polylysine (PLL), gelatin, dextran with a molecular weight of 70 kD, chitosan, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) and 1,1-carbonyldiimidazole (CDI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemical agents were purchased from Sangon Biological Engineering Technology & Services Co. (Shanghai, China) and used without further purification.

Cationic gelatin was synthesized using a previously reported method [12]. Briefly, ethylenediamine and EDC were added to 100 mM phosphate-buffered solution (pH 5.0) containing gelatin at a molar ratio of 10. The pH of the solution was then adjusted to 5.0 by adding 5 M HCl solution. The reaction mixture was agitated at 37°C for 24 hrs and then dialysed against double-distilled water for 48 hrs at room temperature. The dialysed solution was freeze-dried to obtain samples of cationic gelatin. The percentage of amino groups introduced into the cationic gelatin was determined by a conventional trinitrobenzene sulfonate method [13].

C-dextran was synthesized according to a previously reported method with minor modifications [14]. Ethylenediamine and CDI were added to dehydrated dimethyl sulfoxide containing dextran. Following agitation with a magnetic stirrer at room temperature for 24 hrs, the reaction mixture was dialysed against ultra-pure double-distilled water for 2 days using a dialysis membrane with a molecular weight cut-off of 12,000–14,000 (Viskase Companies Inc., Willowbrook, IL, USA). The dialysed solution was freeze-dried to obtain samples of ethylenediamine-introduced dextran (C-dextran). Different degrees of cationization were obtained by changing the quantity of CDI used in the preparations. The cationic degrees were determined by element analysis. The synthetic scheme and the characterization of C-dextran by FT-IR were in supplemental data.

Mouse strains

Female wild-type SD rats and C57/Bl6 mice were purchased from the Experimental Animal Center of Nanjing Medical School (Nanjing, China). IFN-γ receptor-deficient (IFN-γ $R^{-/-}$) mice on the C57/Bl6 background were bred in-house using breeding pairs that were originally purchased from The Model Animal Research Center of Nanjing University (Nanjing, China). All animals were maintained under barrier conditions, and were pathogen-free as assessed by regular microbiologic screening. Macrophage depletion *in vivo* was carried out according to a conventional method using clodronate encapsulated in liposomes [15].

Induction and assessment of rat and mouse AIA

Experiments were performed in 7–8-week-old male rats and mice. All experimental procedures were performed in accordance with the local policy for animal experiments. For statistical analysis, a minimum of 10 animals per group were used, and all experiments were performed in triplicate to ensure the reproducibility of the responses. Rat and mouse AIA was induced according to a previously reported method [16]. Briefly, animals received a subcutaneous injection of Freund's complete adjuvant (CFA) into their footpads (100 μl/rat and 20 μl/mouse).

Animals were evaluated daily for arthritis development. Arthritis was assessed by the clinical scores of the injected feet. The clinical scores ranged from 0–4 according to the degrees of inflammation, with 0 representing no inflammation and 4 representing the most severe inflammation [16].

Therapeutic agents (cationic materials, control materials and MTX) were given every 2 days at the dose of 1 mg/kg body weight *via* an intraperitoneal injection.

Histological assessment

Animals were killed at various times after the induction of arthritis. The knee joints were dissected, fixed in neutral-buffered formalin and embedded in paraffin. Serial sections (5-μm thickness) were cut and stained with haematoxylin and eosin. The stained sections were scored by three independent observers who were blinded to the experimental procedure. The sections were graded subjectively using various parameters. Synovial hyperplasia (pannus formation), cellular exudate, Synovial infiltration was also scored from 0 to 4 and cartilage depletion/bone erosion were each scored from 0 (normal) to 4 (severe). All parameters were subsequently summed to provide an arthritis index (AI; expressed as the mean ± S.E.M.).

Neutrophils in the joints (sectioned from paraffin-embedded tissues) were stained by an alkaline phosphatase staining method using an Alkaline Phosphatase Staining Kit (Jiancheng Biotech, Nanjing, China).

Immunohistochemistry

Paraffin-embedded knee sections were prepared and stained as previously described [17]. Briefly, sections were microwaved twice in 10 mM citrate buffer (pH 6.2) for 10 min. each time. Endogenous peroxidase and biotin were blocked using Chem-Mate Peroxidase Blocking Solution (Sangon) and a Biotin-Blocking System (Sangon), respectively. Next, the sections were incubated with rabbit anti-rat IL-12 P40 (1:50; Boster, Wuhan, China) or rabbit anti-rat IFN-γ (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies. Bound antibodies were detected using an appropriate biotin-conjugated secondary antibody followed by StreptAB Complex (Boster). Sections were developed using diaminobenzidine substrate and counterstained with haematoxylin. Images were captured and analysed using a TE2000U light microscope (Nikon, Milton Keynes, UK). At each time-point examined, the illumination voltage, camera setup and calibration parameters were kept constant for the determination of IL-12 P40 and IFN-γ.

Preparation and analysis of spleen cells and peritoneal macrophages

Spleen cells and peritoneal macrophages were prepared according to conventional protocols [18] and cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (Gibco, Carlsbad, CA, USA). Cells in 6-well plates (1×10^6 cells/well) were stimulated with cationic materials for 24 hrs at a concentration of 100 μg/ml. Supernatants were collected and cytokines were quantified using an ELISA kit (eBioscience, San Diego, CA, USA).

Statistical analysis

Data were analysed by one-way ANOVA followed by Tukey's test as a *post hoc* test. Values of $P < 0.05$ were considered significant.

Results

Amelioration of inflammation by cationic materials in AIA

Rats developed serve inflammation after injection of CFA. Cationic materials were given at 1 hr after the CFA injection. As shown in Fig. 1A, the scores for the inflamed feet revealed that most of the cationic materials used had the ability to suppress the development of adjuvant-induced acute foot inflammation. Within 7 days, the most effective cationic materials were PEI and C-dextran with a cationic degree of 10.2%. Figure 1(B)–(E) shows haematoxylin and eosin stained tissue sections from AIA models without cationic material treatment or treated with PLL, PEI and C-dextran (10.2% modified), respectively, at day 7 after induction of inflammation and administration of cationic materials. Obvious inflammatory cell infiltration was observed in the AIA model without treatment (Fig. 1B). When animals were treated with PLL, PEI or C-dextran, the numbers of infiltrated inflammatory cells were dramatically reduced (Fig. 1C–E). C-dextran almost completely inhibited the development of inflammation (Fig. 1E).

Cationic degrees influence the anti-inflammatory ability of C-dextran

The cationic degrees of C-dextran were altered by changing the quantity of CDI used in the preparation of modified dextran. This facilitated investigations into whether the cationic character of the materials provides their ability to ameliorate the inflammation induced in animal foot pads by adjuvant administration. Figure 2(A) shows the effects of C-dextran with different cationic degrees on AIA models at day 7 after administration of C-dextran. By using unmodified dextran and MTX as controls, an obvious correlation between the cationic degrees and their inhibitory effects on AIA development was observed. Surprisingly, C-dextran with a modification degree of 10.2% had a much better anti-inflammatory ability than MTX. To confirm this observation, the effects of C-dextran (10.2% modified) and MTX in the models were compared over 9 days (Fig. 2B). The effects of C-dextran exceeded those of MTX. Figure 2(A)–(F) shows photos of the affected feet in AIA animals without treatment or treated with non-modified dextran, C-dextran (10.2% modified) and MTX at 7 days after injection with the corresponding medications. Figure 2(G)–(J) shows haematoxylin and eosin stained sections of footpad tissues from the feet shown in Fig. 2(C) and (D), respectively. C-dextran was observed to have better anti-inflammatory effects than MTX at this animal model in all corresponding images.

Cytokine expression profiles of spleen cells and peritoneal macrophages simulated by cationic materials

Rat peritoneal cells (1×10^6) were stimulated with 100 μg/ml of unmodified dextran, PEI or C-dextran (10.2% modified). The concentrations of IL-12, TNF-α, IL-6 and IFN-γ in the supernatants were determined by ELISA. As shown in Fig. 3A, IL-12 was obviously up-regulated by the addition of PEI and C-dextran, while TNF-α, IL-6 and IFN-γ expressions were not changed by the cationic materials. When the same experiments were carried out in spleen cells, up-regulation of IL-12 expression was also observed, as well as a surprisingly high expression level of IFN-γ (Fig. 3B). The concentrations of IFN-γ and IL-12 in animal serum samples changed accordingly following C-dextran injection (Fig. 3C), although the changes were not as large as those in the cellular

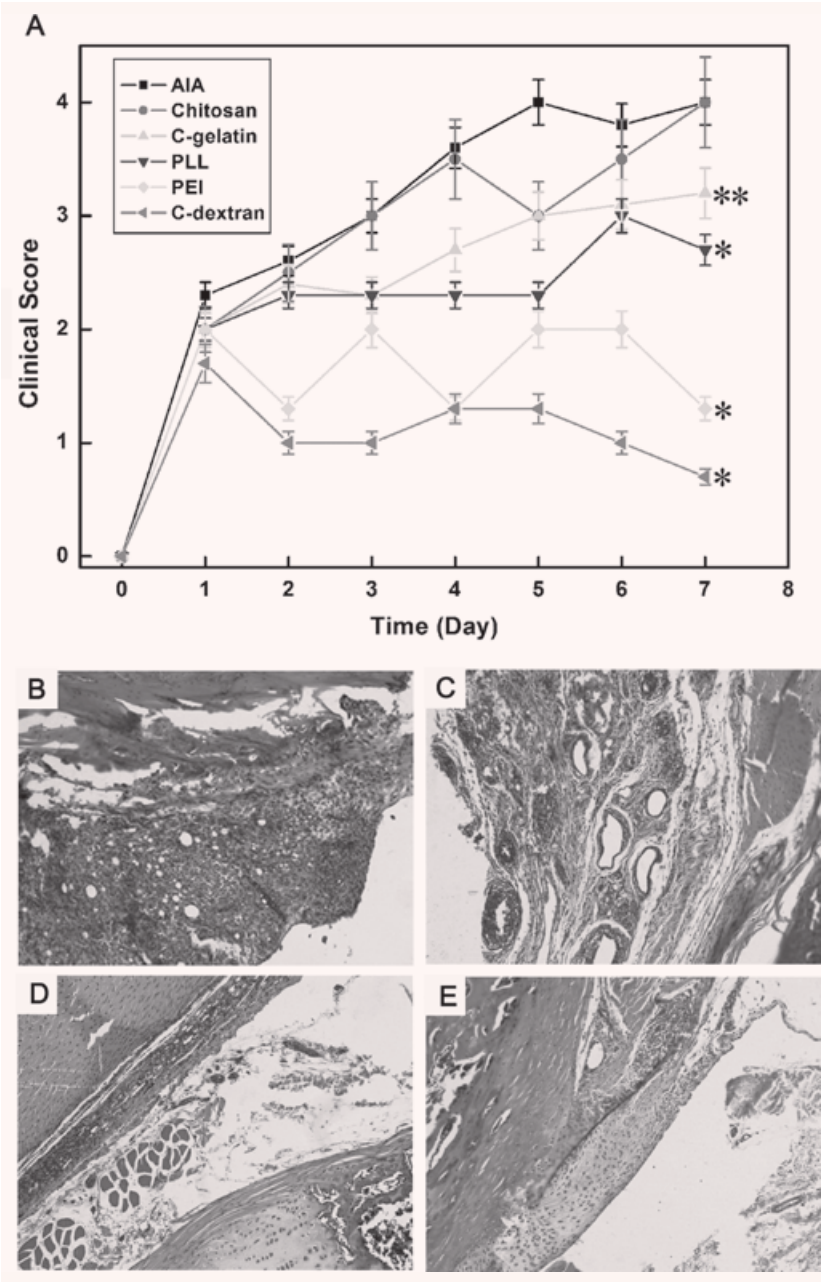

Fig. 1 Amelioration of inflammation by cationic materials in AIA. (**A**) Different cationic materials exhibit significant suppressive effects on AIA inflammation. Cationic materials were given at 1 hr after CFA was injected and the clinical score of each inflammatory footpad was evaluated every day in a double-blinded manner ($n =$ 12 at each time-point, with the experiments carried out in triplicate). Values are the mean and S.E.M. $*P < 0.005$; $**P < 0.05$, *versus* AIA models without cationic material treatment. (**B**) Representative haematoxylin and eosin stained section demonstrating the histopathology of an AIA rat footpad at 3 days after arthritis induction, showing massive synovial and inflammatory cell infiltration. (**C**)–(**E**), Corresponding sections from AIA models treated with PLL (**C**), PEI (**D**) and C-dextran (**E**). (Original magnification $\times$100.)

experiments. The concentrations of the two inflammatory cytokines, TNF-α and IL-6, were not altered in the serum samples (Fig. 3C). Additionally, when tested in macrophage-depleted animals, stimulation of the expression of IL-12 and IFN-γ was greatly weakened (Fig. 3C). Immunohistochemical examinations of IL-12 and IFN-γ in spleen sections revealed that C-dextran also stimulated the expressions of these two cytokines in the spleens (Fig. 3D, E).

IFN-γ and macrophages play critical roles in the amelioration of AIA by cationic materials

To determine whether IFN-γ plays a key role in the effects of the cationic materials on AIA, IFN-γ $R^{-/-}$ mice were used to examine the effects of C-dextran. IFN-γ $R^{-/-}$ mice were highly susceptible to the development of AIA induced by CFA. However, the inflammation

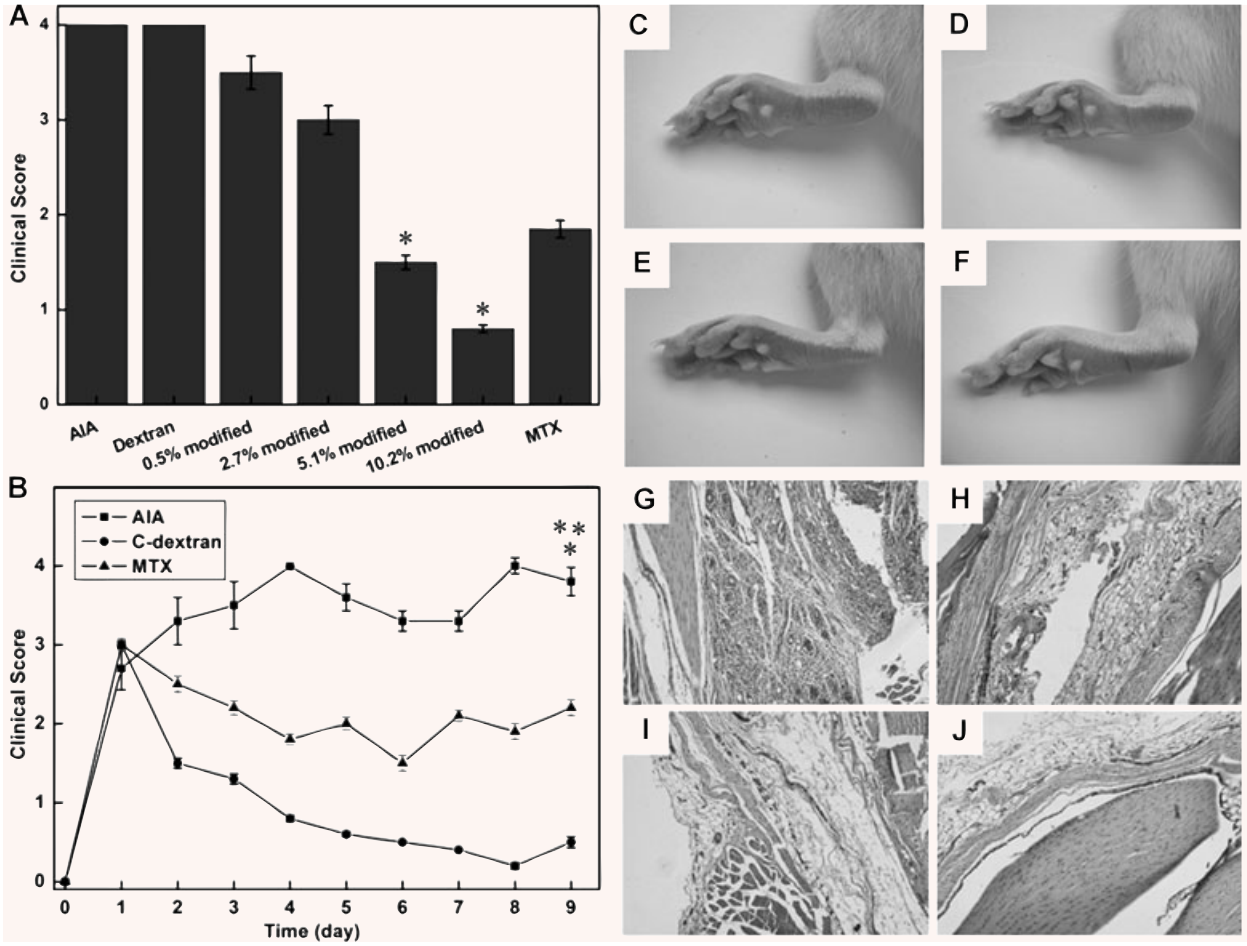

Fig. 2 Cationic degrees influence the anti-inflammatory activity of C-dextran. (**A**) Anti-inflammatory effects of C-dextran with different cationic degrees on AIA animals compared with control animals administered MTX or non-modified dextran ($n = 12$ at each time-point, with the experiments carried out in triplicate). Values are the mean and S.E.M. $^{*}P < 0.005$, *versus* AIA models without cationic material treatment. (**B**) Comparison of the anti-inflammatory activities of C-dextran and MTX. Values are the mean and S.E.M. $^{*}P < 0.005$, *versus* AIA models without cationic material treatment; $^{**}P < 0.001$, *versus* AIA models with MTX treatment. (**C**) Representative footpad of an AIA animal without additional treatment, showing serious swelling caused by inflammation. (**D**)–(**F**) Corresponding footpads from AIA animals treated with non-modified dextran (**D**), 10.2% modified dextran (**E**) and (**F**). (**G**)–(**J**), haematoxylin and eosin stained sections from (**C**), (**D**), (**I**) and (**J**), respectively. (Original magnification ×100.)

in the IFN-γ $R^{-/-}$ mouse model of AIA was not suppressed by C-dextran (Fig. 4A). Consistent with the experiments in SD rats, AIA in wild-type mice was dramatically ameliorated by C-dextran (Fig. 4A). Figure 4(C)–(F) shows photographs of the mouse feet at day 7 of the experiment. Swelling associated with AIA in IFN-γ $R^{-/-}$ mice was not relieved by C-dextran (Fig. 4F), unlike the case in wild-type mice (Fig. 4D).

Macrophage-depleted rats were used to determine the role of macrophages in the pharmacologic mechanisms of the cationic materials. As shown in Fig. 4B, macrophage-depleted rats did not respond to C-dextran administration in a similar way to normal rats. Figure 4(G)–(J) shows photographs of feet from the AIA animal models with and without C-dextran treatment. The swelling of the footpad was not ameliorated by injection of C-dextran when macrophages were depleted (Fig. 4J).

Neutrophil infiltration at inflammatory sites is inhibited by C-dextran-stimulated IFN-γ

IFN-γ $R^{-/-}$ mice were used to investigate neutrophil infiltration at inflammatory sites. After AIA induction, the animals were injected with C-dextran. After 3 days, the inflamed footpads were sectioned and the neutrophil infiltrations were examined by alkaline phosphatase staining. As shown in Fig. 5, massive neutrophil infiltration was observed in the footpads of the AIA models without treatment

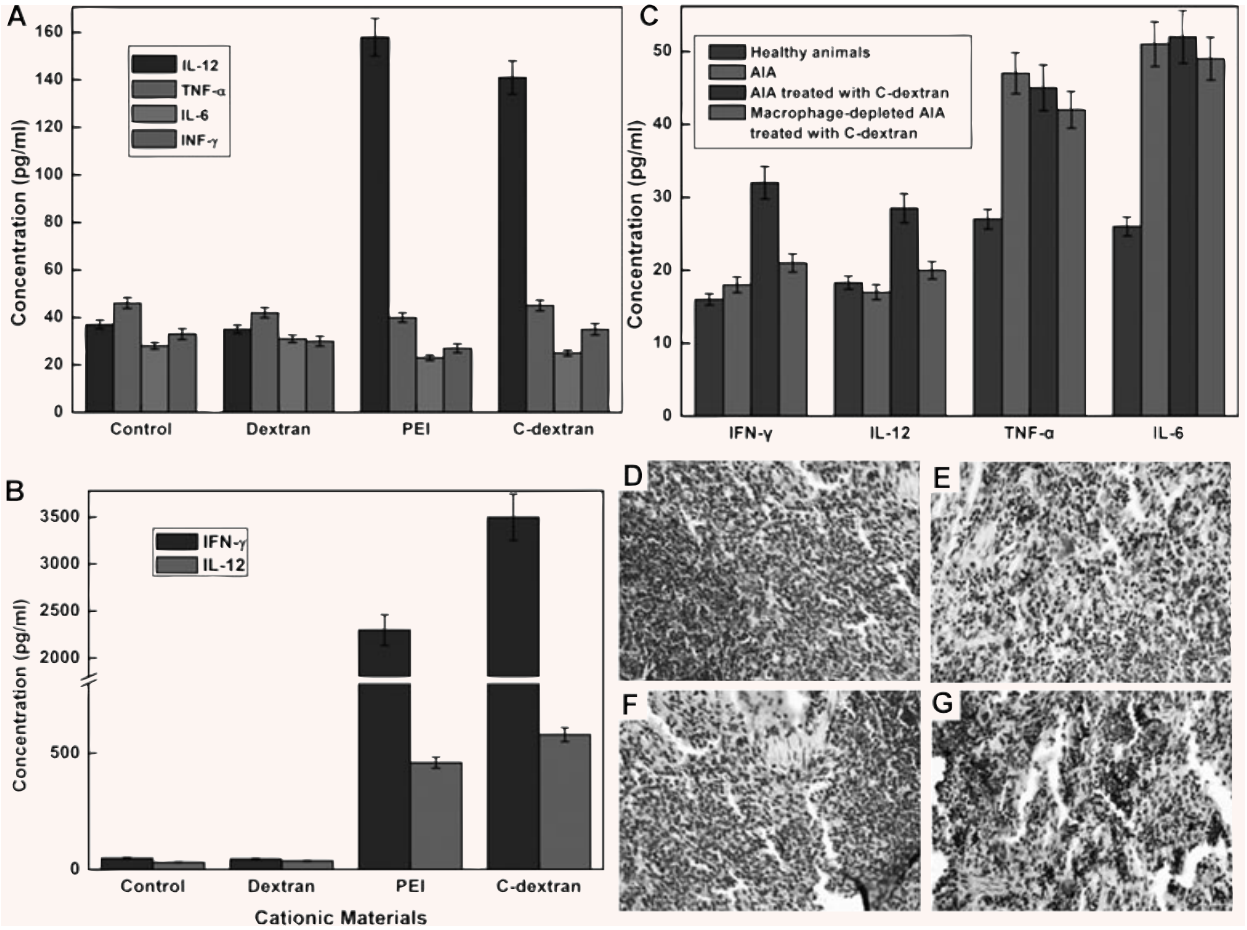

Fig. 3 Cytokine expressions in response to stimulation by cationic materials. (**A**) Expression levels of IL-12, TNF-α, IL-6 and IFN-γ in peritoneal cells stimulated by PEI and C-dextran ($n = 3$ for each sample, with the experiments carried out in triplicate). Values are the mean and S.E.M. (**B**) Expression levels of IL-12 and IFN-γ in spleen cells stimulated by PEI and C-dextran ($n = 3$ for each sample, with the experiments carried out in triplicate). Values are the mean and S.E.M. (**C**) Serum concentrations of IFN-γ, IL-12, TNF-α and IL-6 in normal animals, AIA animals, AIA animals treated with C-dextran and macrophage-depleted AIA animals treated with C-dextran ($n = 7$ for each sample, with the experiments carried out in triplicate). Values are the mean and S.E.M. (**D**), (**E**) Immunohistochemical examination of IL-12 in sections from animals without (D) or with (E) C-dextran treatment. (**F**), (**G**) Immunohistochemical examination of IFN-γ in sections from animals without (**F**) or with (**G**) C-dextran treatment (Original magnification ×600.)

(Fig. 5A, D). When treated with C-dextran, the neutrophils were greatly reduced in the footpads of wild-type mouse AIA models (Fig. 5B). However, in IFN-γ $R^{-/-}$ mouse AIA models, the infiltration of neutrophils did not show any remission (Fig. 5E). Figure 5(C) and (F) shows control samples from a normal wild-type mouse and an IFN-γ $R^{-/-}$ mouse.

Discussion

Cationic materials have been used as gene delivery carriers for decades [19]. Cationic liposomes and cationic polymers are the most commonly used materials [20]. In 1997, Filion *et al.* [21] reported the anti-inflammatory activity of cationic lipids in an arthritis animal model of carrageenan and sheep red blood cell challenge. They proved that the anti-inflammatory activity of the lipids was derived from their cationic components. In our study using cationic polymers as gene delivery carriers, a similar phenomenon was observed. Different cationic polymers, including PEI, cationic peptides and cationic polysaccharides, exhibited evident anti-inflammatory activities in animal models of AIA in the footpad. These results suggest that arthritis-related anti-inflammatory activity may be a universal character of cationic materials.

To investigate the influence of the cationic elements of the materials on their anti-inflammatory activity, C-dextran with different cationic degrees was used. Dextran can be easily modified in

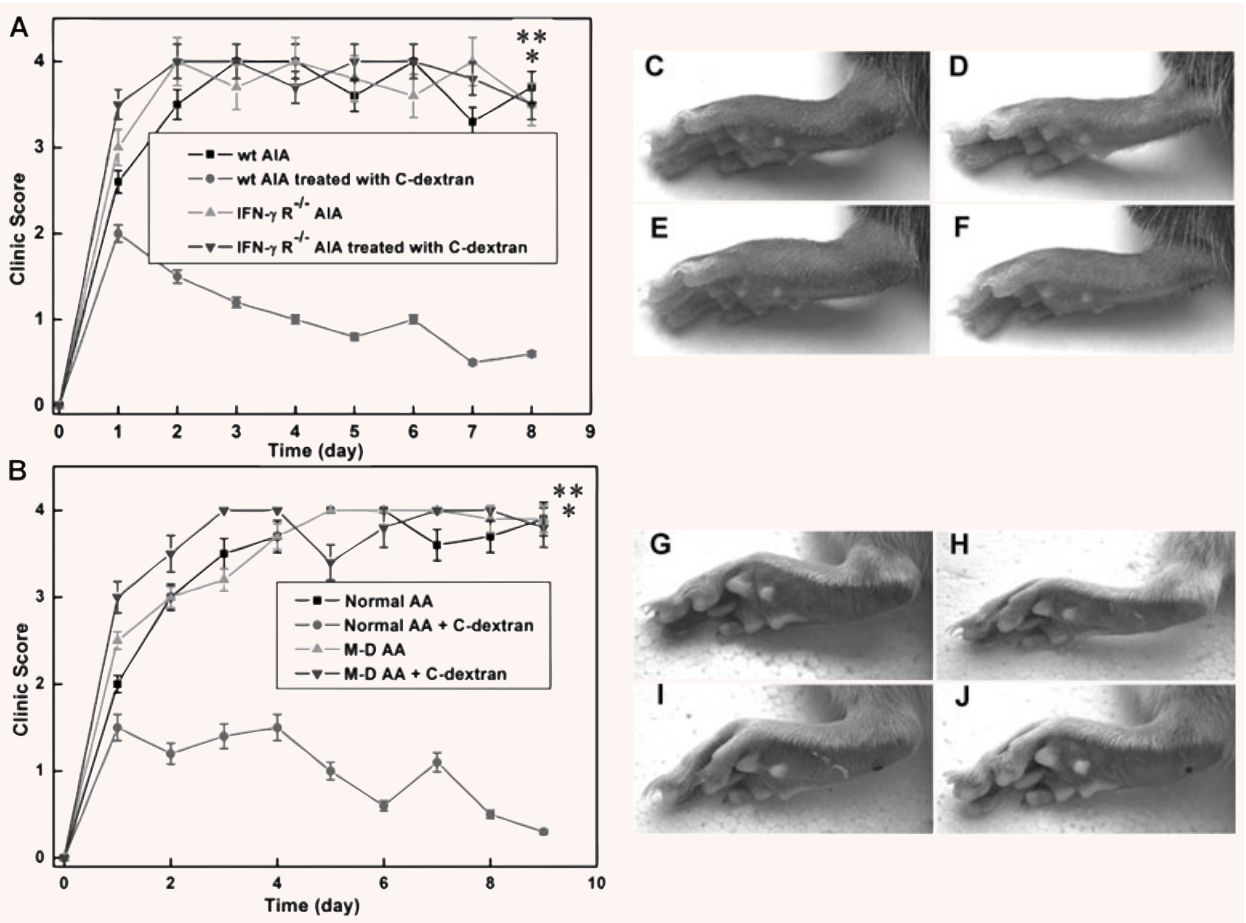

Fig. 4 IFN-γ and macrophages play critical roles in the amelioration of AIA by cationic materials. (**A**) Anti-inflammatory activity of C-dextran in AIA models based on IFN-γ $R^{-/-}$ mice compared with control wild-type mice ($n = 7$ for each sample, with the experiments carried out in triplicate). Values are the mean and S.E.M. $*P > 0.1$, *versus* AIA models based on IFN-γ $R^{-/-}$ mice without C-dextran treatment; $**P < 0.005$, *versus* AIA models based on wild-type mice with C-dextran treatment. (**B**) Anti-inflammatory activity of C-dextran in AIA models based on macrophage-depleted rats relative to control AIA models in normal rats ($n = 7$ for each sample, with the experiments carried out in triplicate). Values are the mean and S.E.M. $*P > 0.1$, *versus* AIA models based on macrophage-depleted rats without C-dextran treatment; $**P < 0.005$, *versus* AIA models based on normal animals with C-dextran treatment. (**C**, **D**) Representative footpads of AIA models in wild-type mice without (C) or with (D) C-dextran treatment. (**E**, **F**) Footpads of AIA models in IFN-γ $R^{-/-}$ mice without (**E**) or with (F) C-dextran treatment. (**G**, **H**) Footpads of AIA models in normal rats without (**G**) or with (**H**) C-dextran treatment. (**I**, **J**) Footpads of AIA models in macrophage-depleted rats without (**I**) or with (**J**) C-dextran treatment.

a gradient manner, which provides the convenience of excluding the influences of other factors, such as the chemical structure, physical state, molecular weight and hydrophilic/hydrophobic properties. In addition, C-dextran has very low toxicity. When used in cellular studies, it does not influence the growth and activities of cells, and this guarantees that the stimulatory effects are not derived from the process of cell apoptosis. The present results indicated that the cationic degree directly altered the anti-inflammatory activity of C-dextran. Non-modified dextran had no effect on the inflammation. C-dextran with a modification of 10.2% was the most effective, and its anti-inflammatory effect was better than that of MTX at the same dose.

To clarify why cationic materials have this anti-inflammatory activity, cells (peritoneal macrophages and spleen cells) involved in immune reactions were used to examine the cytokine expressions in response to challenge by cationic materials. Cationic materials can combine anionic proteins in the tissue fluid and serum [22]. The complexes formed by cationic materials and proteins are captured and eliminated from body fluids by macrophages [23]. Therefore, macrophages are the first cells activated by cationic materials and respond by secreting certain cytokines. Because intraperitoneal administration was used in the present study, peritoneal macrophages were chosen for these experiments. Our results revealed that

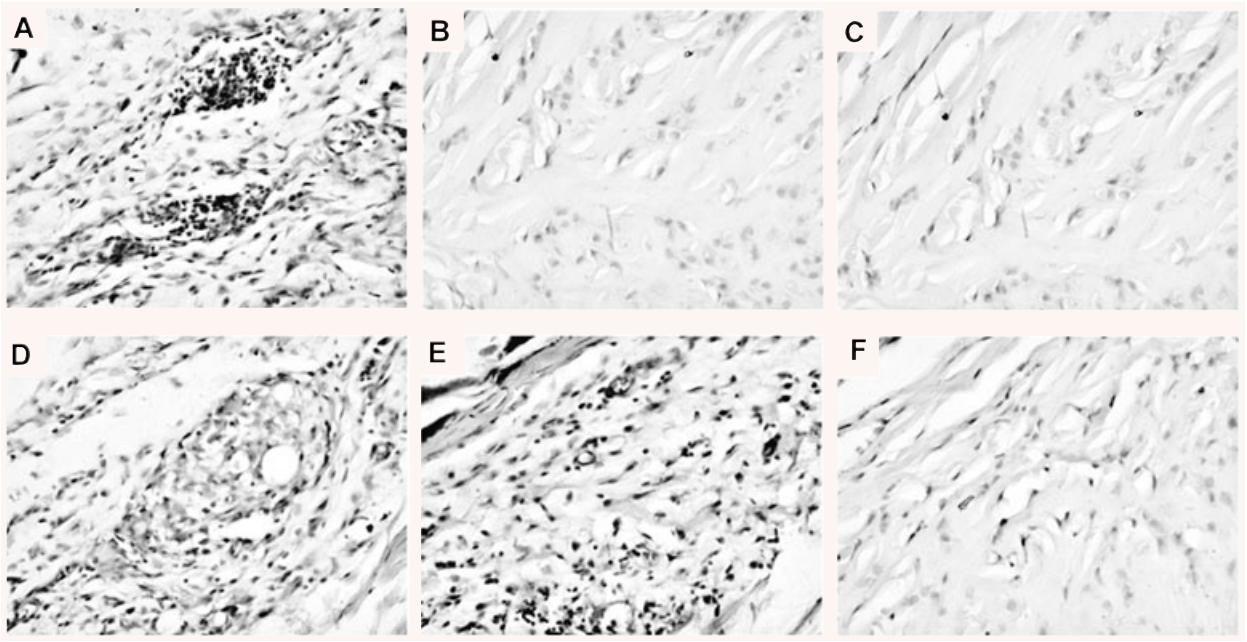

Fig. 5 Neutrophil infiltration at inflammatory sites is inhibited by C-dextran. (**A**) Alkaline phosphatase-stained sections of footpads from AIA models in wild-type mice. (**B**) Corresponding section from C-dextran-treated animals. (**C**) section from controlling animals. (**D**) Alkaline phosphatase-stained sections of footpads from AIA models in IFN-γ $R^{-/-}$ mice. (**E**, **F**) Corresponding sections from C-dextran-treated (**E**) and healthy (**F**) animals (experiments were carried out in triplicate). (Original magnification ×600.)

macrophages stimulated by cationic materials up-regulated their expression of IL-12.

IL-12 is an important cytokine in immune reactions, and has the ability to activate NK cells towards secretion of IFN-γ [24]. IFN-γ has been intensively studied for its possible anti-arthritis activity [5–8]. Spleen cells were used to examine the stimulation by cationic materials, because they comprise a mixture of multiple cell types including T cells, B cells, NK cells and macrophages [25]. When challenged with cationic materials, up-regulation of IL-12 expression was detected and subsequent high expression of IFN-γ was observed. When tested *in vivo*, the serum concentrations and spleen expressions of IL-12 and IFN-γ were also elevated when animals were given C-dextran, although the up-regulation was less than that in the *in vitro* experiments.

In a study by Wu *et al.* [8] regarding CpG DNA-stimulated IFN-γ inhibition of experimental arthritis, the authors demonstrated that the inhibitory effect of IFN-γ was due to its ability to prevent neutrophil infiltration from blood into joints by combining with IFN-γ receptors on the surface of neutrophils. This conclusion is consistent with our observations of reduced numbers of neutrophils infiltrating the joints when the animals were treated with C-dextran. Furthermore, this effect of C-dextran was abolished in IFN-γ $R^{-/-}$ mice. Another group reported that IFN-γ could reduce the expression of a neutrophil chemotaxin, CXCL-8, which resulted in decreased neutrophil infiltration at inflammatory sites of an experimental arthritis model [9]. However, we did not found any variation in the expression of CXCL-8 in our tested animals with or without C-dextran treatment (data not shown). This discrepancy may arise because the animal models used in the respective studies were different. IFN-γ is mainly secreted by IL-12-activated NK cells *in vivo*. Our cellular experiments demonstrated that cationic polymers could stimulate macrophages to express IL-12. To verify the role of macrophages *in vivo*, macrophage-depleted rats were used to test the potency of C-dextran. We found that the effect of C-dextran was remarkably weakened when macrophages were depleted.

Combining all the findings of the present study, we propose a mechanism for the anti-arthritis activity of cationic materials (Fig. 6). Cationic materials or cationic material/protein complexes are phagocytized by macrophages. Subsequently, an unknown receptor detects the components and activates the cells to secrete IL-12, which in turn stimulates NK cells to express IFN-γ. The combination of IFN-γ and its receptors on the surface of neutrophils prevents neutrophil infiltration into inflammatory sites. This process eventually relieves the inflammation associated with arthritis. However, the receptor that can detect the materials and transfer the signals of stimulation was not identified in the present study. It may be a certain kind of receptor involved in natural immunity, such as a Toll-like receptor, because macrophages represent one of the main cell lines in natural immunity [26]. When foreign materials are

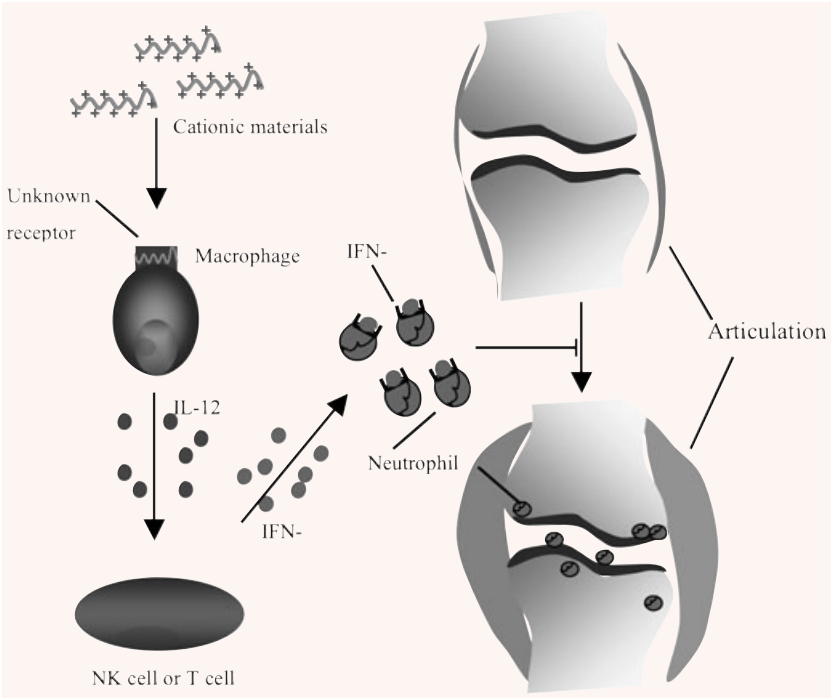

Fig. 6 Possible mechanisms involved in the anti-inflammatory activity of cationic materials. Cationic materials signal through an unknown receptor on macrophages to induce IL-12 secretion. In turn, the secreted IL-12 promotes the expression of IFN-γ by NK cells (or T cells). The resulting elevated systemic levels of IFN-γ inhibit arthritis development by preventing neutrophil recruitment to inflammatory sites.

injected into the body, they are easily detected by receptors that mainly function in natural immunity. Additional studies are required to identify the receptor before a conclusion can be reached regarding our proposed mechanism.

There are differences among the anti-inflammatory activities of materials, which may arise through differences in their molecular weights, cationic extents, solubilities and biocompatibilities. In our study, C-dextran exhibited the best effects on suppressing AIA. This may be because the biocompatibility of dextran, especially its *in vivo* behaviour, is excellent. In clinical practice, dextran is usually used in plasma substitutions [27]. It exhibits no cytotoxicity, low physical side effects and high solubility, and is easily digested and discharged from the body. Accordingly, although PEI possesses many more cationic elements, it did not show better efficacy than C-dextran, which may be because it is non-biodegradable and has relatively high toxicity [28].

IL-12 and IFN-γ are important cytokines in the immune system. They can stimulate other cells to secret a batch of inflammatory cytokines, including TNF-α and IL-6 [29]. Although we did not detect any obvious changes in the circulating concentrations of such cytokines, we did observe up-regulation of such cytokines in experiments on spleen cells (data not shown). Hydroperitoneum and intestinal adhesion caused by long-term repeated use of cationic materials at high doses were observed in our studies, and these side effects were reduced when the injections of the cationic materials were stopped. Some cationic materials, such as PEI, exhibit significant cytotoxicity, which can cause obvious cell death [30]. These types of material-caused inflammation may be due to the cytotoxicity of the materials. The C-dextran used in the present study does not show serious toxicity towards cells, but was still associated with the above side effects. These observations imply that there must be some specific physical toxicity (such as renal toxicity that can cause hydroperitoneum) that is directly related to the cationic character of the materials.

In conclusion, different cationic materials share the property of suppressing arthritis-related inflammation. The present findings suggest that this property may originate from their ability to stimulate the secretion of IL-12 and subsequent secretion of IFN-γ. Up-regulated IFN-γ prevents the infiltration of neutrophils into inflammatory sites, which results in remission of the inflammation caused by experimental arthritis. The findings provide some novel candidates for anti-RA drug discovery and the potential to apply medications derived from cationic materials to clinical practice.

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