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Original Article

A dual fluorescent reverse targeting drug delivery system based on curcumin-loaded ovalbumin nanoparticles for allergy treatment

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Abstract

A reverse targeting drug delivery based on antigen-modified nanoparticles provided an innovative strategy for effectively alleviating or inhibiting immune response. In this study, a dual fluorescent reverse targeting drug delivery system based on curcumin-loaded ovalbumin nanoparticles is developed for allergy treatment. The self-crosslinked ovalbumin nanoparticles achieved the double function of reverse targeting and sustained delivery carriers to maximize the anti-allergy of curcumin. Using a murine model of ovalbumin-induced allergy, this drug delivery system suppressed antigen-specific IgG1 and IgE production, inhibited CD4⁺ T activity, and decreased the level of ovalbumin-sensitized memory B cells. The curcumin-loaded ovalbumin nanoparticles exert stronger and more effective treatment on the immunomodulatory role. Furthermore, fluorescence imaging in vivo can reveal the precise delivery process for the effective allergy immunotherapy. The dual fluorescent reverse targeting delivery system provided a significant potential for the visible treatment of allergy with the merged functions of targeting, vehicles and fluorescence.

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Key words: Reverse targeting; Allergy; Ovalbumin nanoparticles; Curcumin; Fluorescence imaging; Drug delivery

Allergic diseases represent a significant public health problem worldwide, which increases significantly in the prevalence of systemic immunological diseases. Drug therapy is crucial for allergy treatment; however, a reduction of systemic side effects is urgently required.¹ Curcumin exhibits a variety of pharmacologic activities in various allergies (including asthma).^{2,3} Furthermore, curcumin might attenuate asthmatic activity via the inhibition of nuclear factor- κ B activation.⁴ However, previous studies have revealed that curcumin treatment has been challenged for clinical efficacy and bioavailability owing to its extremely low

aqueous solubility, slow gastrointestinal absorption, and rapid metabolism.⁵ Nanoparticles-based drug delivery systems have shown major progress for the novel treatment of allergy in recent years.^{6–9} Given that allergies are currently difficult to completely cure, the design and development of a nano delivery system could conceivably be applied to allergy immunotherapy.^{8,10} A nano drug delivery system that delivers curcumin to the inflamed areas with prolonged release would initiate strategies to improve allergy treatment.^{11–13}

To alleviate or inhibit immune response effectively, an anti-allergic agent must be delivered to target immune cells such as mast cells. A reverse targeting drug delivery system based on antigen-modified nanoparticles provided an innovative strategy for allergy treatment.¹⁴ In this delivery system, ovalbumin (OVA)-modified nanoparticles as drug carriers were adversely recognized and captured by antigen-sensitized immune cells. Hence, more drugs will be targeted and delivered to sensitized cell to generate anti-allergic effects through directly damaging the cells or modulating different signaling pathways. This proved that OVA-modified liposomes could deliver the model drug doxorubicin as a reverse targeting carrier to suppress the

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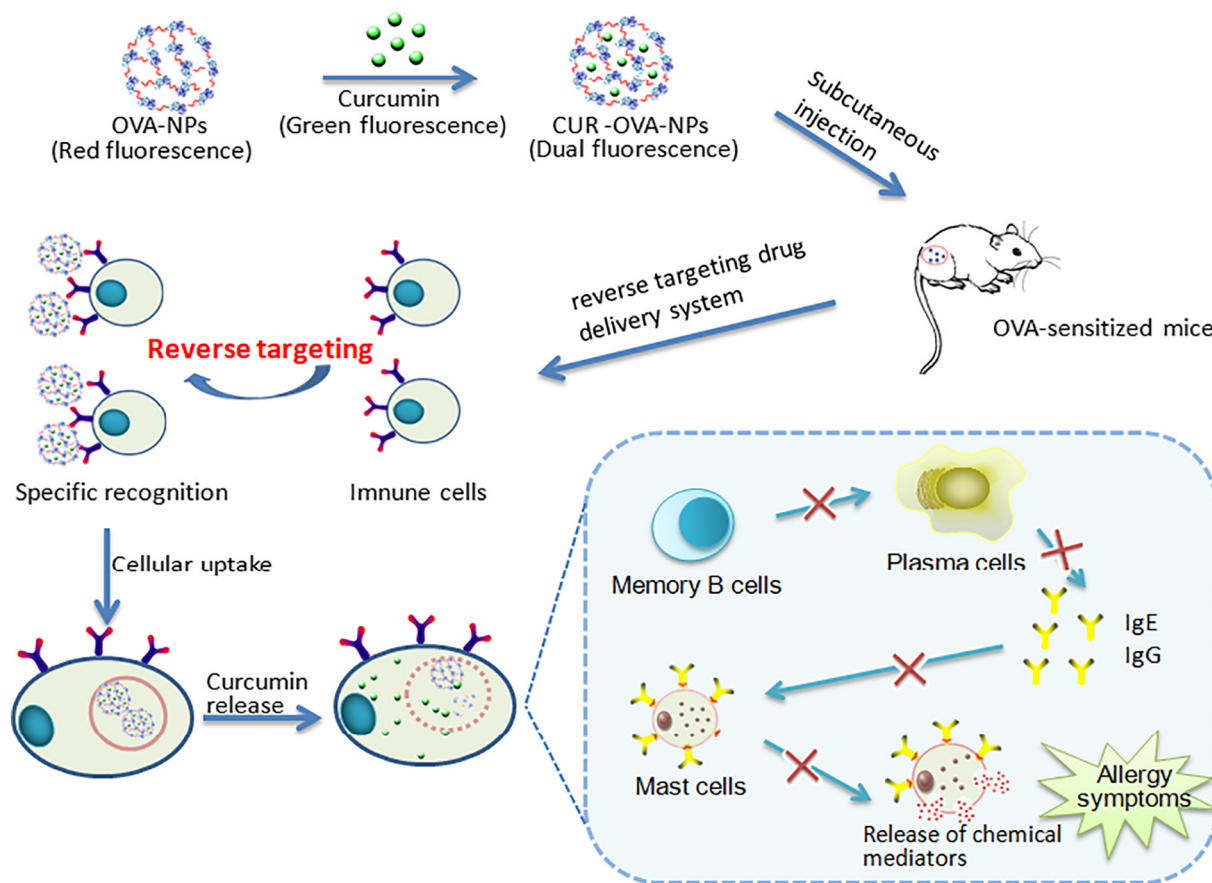


Figure 1. Schematic of reverse targeting curcumin-loaded ovalbumin nanoparticles for allergy treatment.

production of IgE significantly in OVA-sensitized mice, subsequently leading to a hyposensitization effect on immune cells by causing direct damage. Based on this reverse targeting drug delivery system, the self-crosslinked ovalbumin nanoparticles may exert their special advantages for allergic diseases because of their good biocompatibility, defense for the incorporated drug against rapid degradation, and the targeting control for immune cells. The self-crosslinked OVA nanoparticles can achieve the double function of reverse targeting and sustained delivery carriers to maximize anti-allergy effects without additional carriers.

For a drug delivery system, real-time tracking the delivery process in vivo is crucial in revealing the mechanism.^{15–17} A visible dual fluorescent tracking drug delivery system for multispectral combination is demanded owing to its precise real-time control over delivering drugs^{18,19}. Based on multispectral imaging with different excitation wavelengths, a fluorescence drug-loaded carrier was designed to perform real-time monitoring with non-interfering signals. The drug carrying system was tracked in vivo by the fluorescence imaging system, and the noninvasive release of the drug in vivo can be real-time monitored with the sustained delivery of the drug. Recently, researchers have focused more on the dual fluorescence imaging technology of multispectral materials in vivo.^{20,21} In our previous studies, dual fluorescent drug delivery systems have

been reported for tracking the distribution of drugs and the degradation of the carriers via multispectral fluorescence imaging.^{18,22} Multispectral fluorescence imaging tracking can also be applied in the curcumin-loaded delivery system for allergy treatment owing to the visible fluorescence of curcumin, which is a vital avenue to monitor the drug and carrier for underlying disease treatment.

Herein, a dual fluorescent reverse targeting drug delivery system based on curcumin-loaded ovalbumin nanoparticles (CUR-OVA NPs) was developed for allergy treatment (Figure 1). Unlike conventional drug delivery systems that specifically deliver drug molecules to the targeted cells, the reverse targeting drug delivery system will be recognized by specific immune cells especially B cells or mast cells, which are crucial in the occurrence and development of allergic reaction. Through this strategy, the anti-allergic drug curcumin will mediate OVA-sensitized cells by special recognition to OVA nanoparticles in an OVA-sensitized mice model. The production of IgE antibody as the determining factor of allergy would be influenced owing to the effect of curcumin on B cells. Consequently, the release of allergy-causing chemical mediators (such as histamine and leukotrienes from mast cells) would decrease because of the direct effect of curcumin and decreased IgE level. All these effects will ultimately alleviate or inhibit allergy reactions in the body based on the curcumin-loaded reverse targeting drug delivery system. Moreover, based on the

beneficial fluorescence of genipin crosslinked nanoparticles,²³ the loading drug and the OVA nanoparticles delivery system can be monitored accurately and objectively by the multispectral fluorescence separation of genipin-crosslinked carriers and curcumin. Further, the fluorescence intensities can reveal the precise process of the drug and carrier degradation, recording the matured dendritic cells (DCs) migrated to the lymph node for an effective allergy immunotherapy. The reverse targeting drug delivery system of CUR-OVA NPs could suppress a murine model of OVA-induced allergy, exerting a stronger and more effective treatment on the immunomodulatory role.

Methods

Preparation of OVA and BSA-sensitized mice

For active primary sensitization, OVA-sensitized BALB/c mice were immunized intraperitoneally by 100 µg OVA with Imject®Alum on day 0. On day 15, mice were sensitized with the same antigen to construct OVA-sensitized mice. Similarly, BSA-sensitized mice were immunized intraperitoneally by 100 µg of BSA with Imject®Alum.

Equal doses of free OVA and OVA NPs (each containing 100 µg OVA) were injected subcutaneously into BALB/c mice on days 8, 10, and 12 after first sensitizing with PBS treatment as a control. After serum samples were collected on days 7, 14, and 22 post-sensitization, OVA-specific antibodies, IgG1 and IgE, in the serum samples were determined by ELISA.

Treatment of sensitized mice with CUR-OVA NPs

On days 8, 10, and 12 after the first sensitization, the BALB/c mice were subcutaneously injected with the equal doses of free curcumin and CUR-OVA NPs (200 µg curcumin per mouse in each injection) with PBS treatment as a control. The mice were boosted with the same antigen on days 15 and 29. Subsequently, OVA-specific antibodies IgG1 and IgE in the serum samples were determined by ELISA on days 7, 14, and 22 post-sensitization.

Additionally, splenocytes (2×10^6 cells) were harvested from curcumin and CUR-OVA NP-treated groups on days 7, 14, and 22 post-sensitization and restimulated with OVA (50 µg/mL) for 72 h at 37 °C. Subsequently, the cells were collected, pre-incubated with TruStain fcX™ at 1.0 µg per 10^6 cells in 100 µL volume for 10 min on ice, and stained with anti-mouse antibodies against CD3e, CD4, CD19, and CD27 for 30 min at 4 °C. The proportion of CD4⁺ T cells and CD19⁺CD27⁺ memory B cells in the spleen were measured using a flow cytometer (BD Biosciences, San Jose, CA).

The targeting of CUR-OVA NPs

To illustrate the suppressive effect of the targeting nanoparticles of CUR-OVA NPs in contrast to free curcumin, splenocytes (2×10^6 cells) were harvested from BSA-sensitized mice and OVA-sensitized mice 7 days post-sensitization, and subsequently treated with CUR and CUR-OVA NPs (OVA content 20 µg/mL) for 24 h at 37 °C. Subsequently, the cells were collected, pre-incubated with TruStain fcX™ at

1.0 µg per 10^6 cells in 100 µL volume for 10 min on ice, and stained with fluorescein-labeled anti-mouse antibodies cy5.5-anti-CD19, APC-anti-MHCII for 30 min at 4 °C. The proportions of CD19⁺genipin⁺ and CD19⁺MHC II⁺ B cells were analyzed using a flow cytometer (BD Biosciences, San Jose, CA).

Dual fluorescence tracking migration and degradation of free curcumin, OVA NPs, and CUR-OVA NPs

To separate the fluorescence signal of curcumin and OVA NPs, multispectral fluorescence imaging in vitro was performed using an imaging system (Maestro 2,CRI,USA) initially. Subsequently, the migration and degradation of free curcumin, OVA NPs, and CUR-OVA NPs were tracked in vivo using a CRI imaging system with an exposure time of 200 ms at an excitation wavelength of 595 nm to extract the signal from the OVA NPs, and an excitation wavelength of 455 nm to extract the signal from the curcumin. The fluorescence signals were also calculated quantitatively over time using the Maestro software.

Statistical analysis

Results were expressed as mean ± SD, followed by the student's t-test, analysis of variance (ANOVA), and Tukey's post hoc test. P values of 0.05 or less were regarded as statistically significant.

Results

Characterization of nanoparticles, drug release, and cellular toxicity detection

OVA-crosslinked nanoparticles (OVA NPs) were successfully prepared by the genipin-mediated crosslinking of OVA and PEI. As shown in Figure 2, A, the determination of α-helix, beta-pleated sheet, and the turn and random coils are relatively consistent between the free OVA and OVA NPs. The similar spatial structure of free OVA and OVA NPs demonstrated that the crosslinking of OVA NPs would not destroy the structure of OVA. Moreover, AFM was used to investigate the surface features of the samples (Figure 2, B). The OVA NPs and CUR-OVA NPs showed no obvious morphological difference. As shown in Figure 2, C, the mean diameter of the OVA NPs and CUR-OVA NPs are 216.4 nm (PDI = 0.371) and 206.9 nm (PDI = 0.330), respectively. Additionally, the zeta potential of the CUR-OVA NPs (39.8 mv) is slightly higher than that of the OVA NPs (23.9 mv), which was beneficial to cellular uptake. Subsequently, the UV-vis and fluorescence spectra of the OVA NPs and CUR-OVA NPs were performed. In Figure 2, D, the free curcumin and OVA NPs show a characteristic absorption peak at approximately 450 and 600 nm, respectively. There is a weak absorption peak at approximately 450 nm and a strong absorption peak at approximately 600 nm in the UV-Vis spectrum of the CUR-OVA NPs. The fluorescence spectra of OVA NPs and CUR-OVA NPs indicated that the maximum emission of genipin was observed at the emission of 630 nm, with an excitation wavelength at 450 nm (Figure 2, E) and 580 nm (Figure 2, F). Additionally, free curcumin exhibited the maximum emission peak with an excitation wavelength at 450

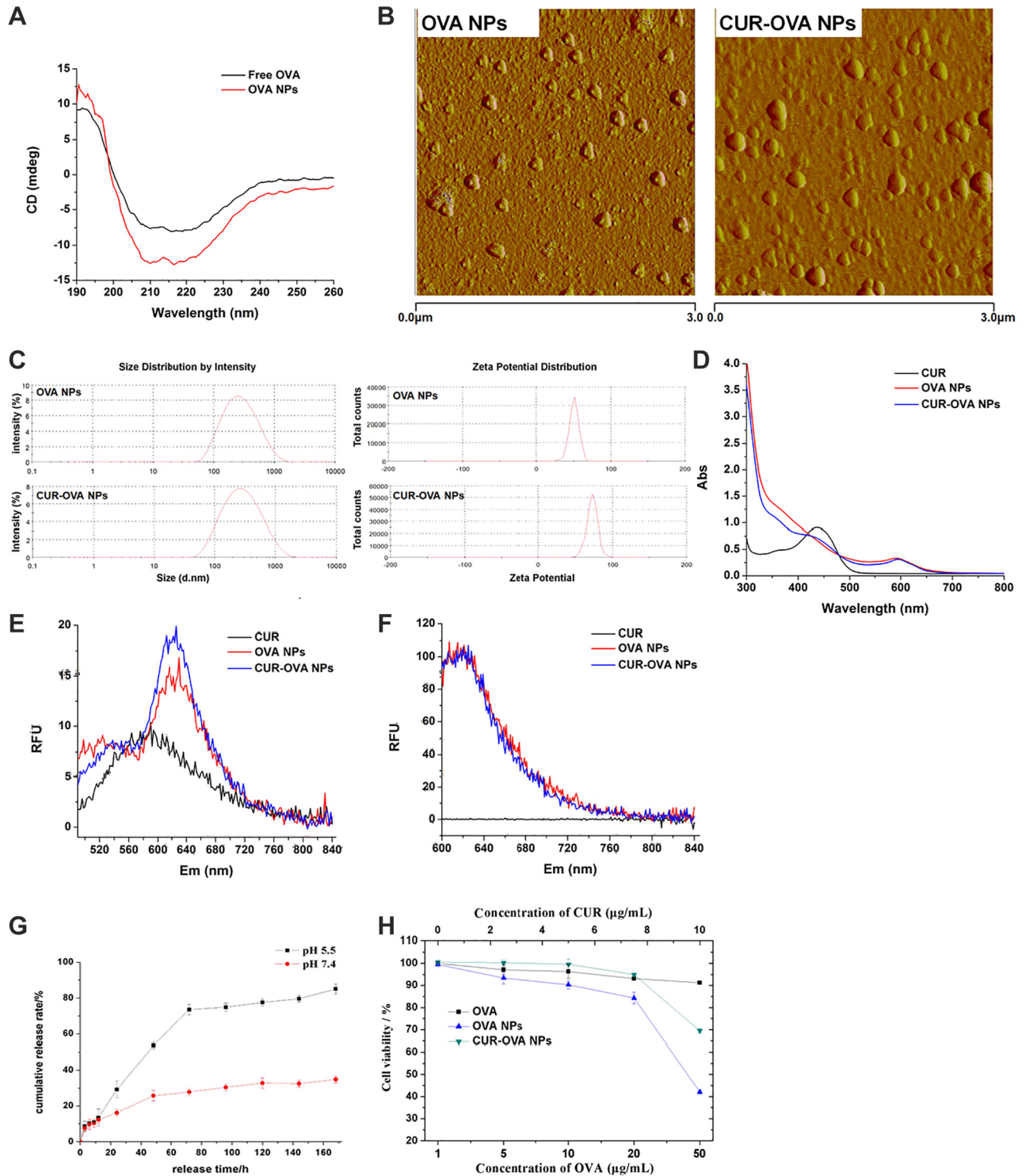


Figure 2. Characterization of CUR-OVA NPs. **(A)** Circular Dichroism Chiroptical Spectra of free OVA and OVA-NPs, **(B)** AFM of OVA NPs and CUR-OVA NPs, **(C)** Size and zeta potential distribution of OVA-NPs and CUR-OVA NPs, **(D)** UV-vis spectra of CUR, OVA NPs and CUR-OVA NPs, **(E)** Fluorescence emission spectra of CUR, OVA NPs and CUR-OVA NPs with an excitation wavelength at 450 nm, **(F)** Fluorescence emission spectra of CUR, OVA NPs and CUR-OVA NPs with an excitation wavelength at 580 nm, **(G)** In vitro release of curcumin from CUR-OVA NPs under buffer conditions (pH 7.4 and 5.5). Data were presented as mean $\pm$ SD, $n = 3$, **(H)** Cellular toxicity of OVA, OVA NPs and CUR-OVA NPs, Data were presented as mean $\pm$ SD, $n = 6$.

nm, but no fluorescence at the excitation wavelength of 580 nm. However, CUR-OVA NPs could scan the maximum emission peak with an excitation wavelength at 580 nm. For in vivo

visualization fluorescence tracking, the optical intensities of OVA NPs and CUR-OVA NPs are important factors. The data above demonstrated that OVA nanoparticles had been

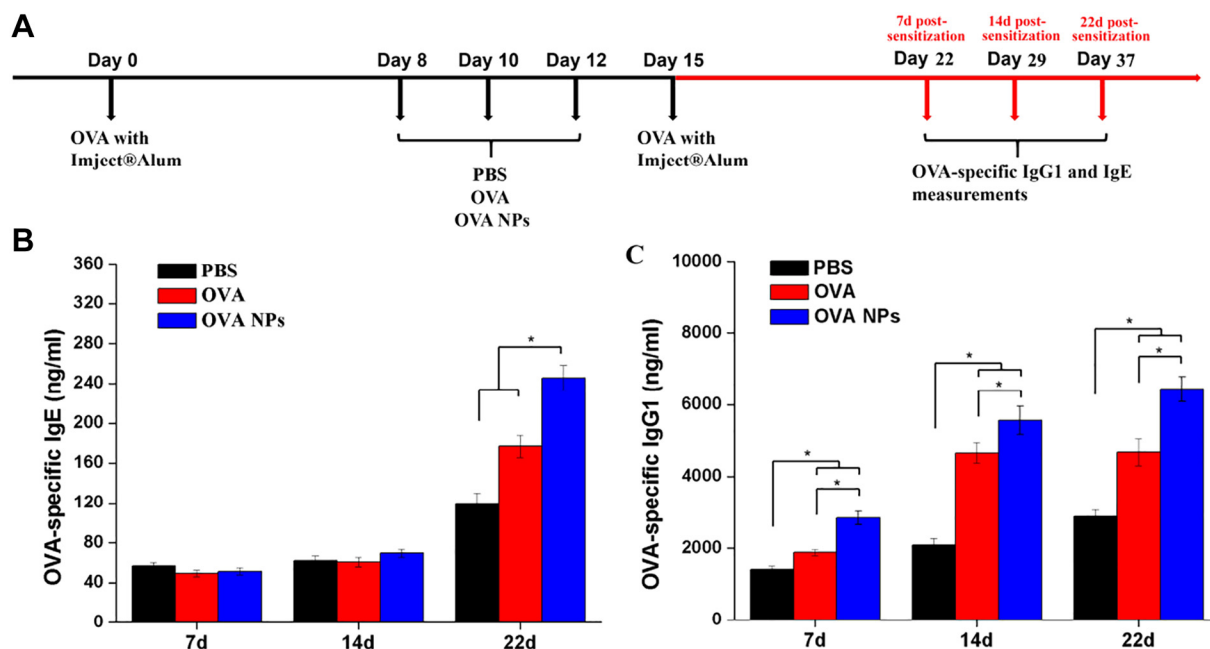


Figure 3. OVA NPs-induced IgE and IgG1 production in OVA-sensitized immune mice. (A) Scheme showing the experiment design to evaluate the amount of OVA-specific IgG1 and IgE in serum of OVA-sensitized mice, (B) The amount of OVA-specific IgE on days 7, 14, and 22 after sensitization, (C) The amount of OVA-specific IgG1 on days 7, 14, and 22 after sensitization. Each point is presented as the mean $\pm$ SD, $n = 6$ (* $p < 0.05$).

successfully constructed and are promising as a visible antigen and drug delivery system.

The encapsulation efficiency and drug-loading rate of CUR-OVA NPs were 79.31% and 19.83%, respectively. The in vitro drug release of curcumin from the CUR-OVA NPs was performed successfully in PBS media with a pH of 7.4 and 5.5 for 7 days. As shown in the release curve (Figure 2, G), a burst release occurred during the first 72 h with the same trend in both pH media. After 7 days, the released curcumin was approximately 34% in the neutral phosphate saline buffer versus approximately 84% in the acid buffer. The release of curcumin was more rapid in phosphate saline buffer at pH 5.5 than at pH 7.4, suggesting that an acid microenvironment is helpful for drug release. These results confirmed the sustained and controlled release from OVA nanoparticles.

The assay for cellular toxicity indicated that free OVA and curcumin did not lead to any cytotoxicity at various concentration, whereas OVA NPs and CUR-OVA NPs generated a certain cytotoxicity on the DCs at the concentration of 50 $\mu\text{g/mL}$ (OVA antigen content) (Figure 2, H). This was because the high positive charge of PEI exerted a toxic effect and inhibited the growth of BMDCs. This result was consistent with our previous report.²⁴ Therefore, 20 $\mu\text{g/mL}$ of OVA NPs and CUR-OVA NPs is a suitable dosage for an in vitro test. Furthermore, the data suggested that the anti-allergic effect of free curcumin and CUR-OVA NPs was not achieved from the direct damage to B cells.

OVA NPs-induced IgE and IgG1 production in OVA-sensitized immune mice

To verify the sensitivity of OVA NPs, the production of OVA-specific IgE and IgG1 were examined in the serum at 7, 14,

and 22 days post-sensitization (Figure 3, A). As shown in Figure 3, B, the antibody production in PBS-treated mice is significantly less than that in OVA- or OVA NP-treated group. The OVA NPs significantly elevated the serum levels of OVA-specific IgG1 after treatment with 100 μg OVA NPs compared to the free-OVA-treated mice on days 7, 14, and 22. For OVA-specific IgE (Figure 3, C), the production increased by approximately 42% on day 22, while no obvious difference was observed between the two groups on days 7 and 14. Because the OVA NPs could be effectively recognized by immune cells in the OVA-sensitized mice, they induced higher productions of IgE and IgG1 with the adjuvant effect of Imject® Alum. The results further suggested that OVA NPs themselves could be potential drug delivery for anti-allergic therapy because of the higher sensitivity of immune cells to OVA NPs. Unlike conventional targeting delivery systems that directly deliver agents to the target cells, the OVA NPs might be first recognized by specific target cells; subsequently, the loading agents were delivered to the immune cells, which was a reverse targeting delivery strategy.

IgE and IgG1 production after treatment with reverse targeting CUR-OVA NPs

To examine whether OVA NPs could be used as a carrier for delivering drugs to the immune cells, curcumin as an anti-allergic drug was encapsulated in the OVA NPs to form CUR-OVA NPs. The inhibiting OVA-specific immune response of CUR-OVA NPs was evaluated by the levels of OVA-specific IgE and IgG1 antibodies on OVA-sensitized BALB/c mice. As shown in Figure 4, both curcumin and CUR-OVA NPs remarkably inhibit the production of OVA-specific antibodies compared with the PBS control. The CUR-OVA NPs significantly suppressed antigen-specific IgE (Figure 4, B) and IgG1

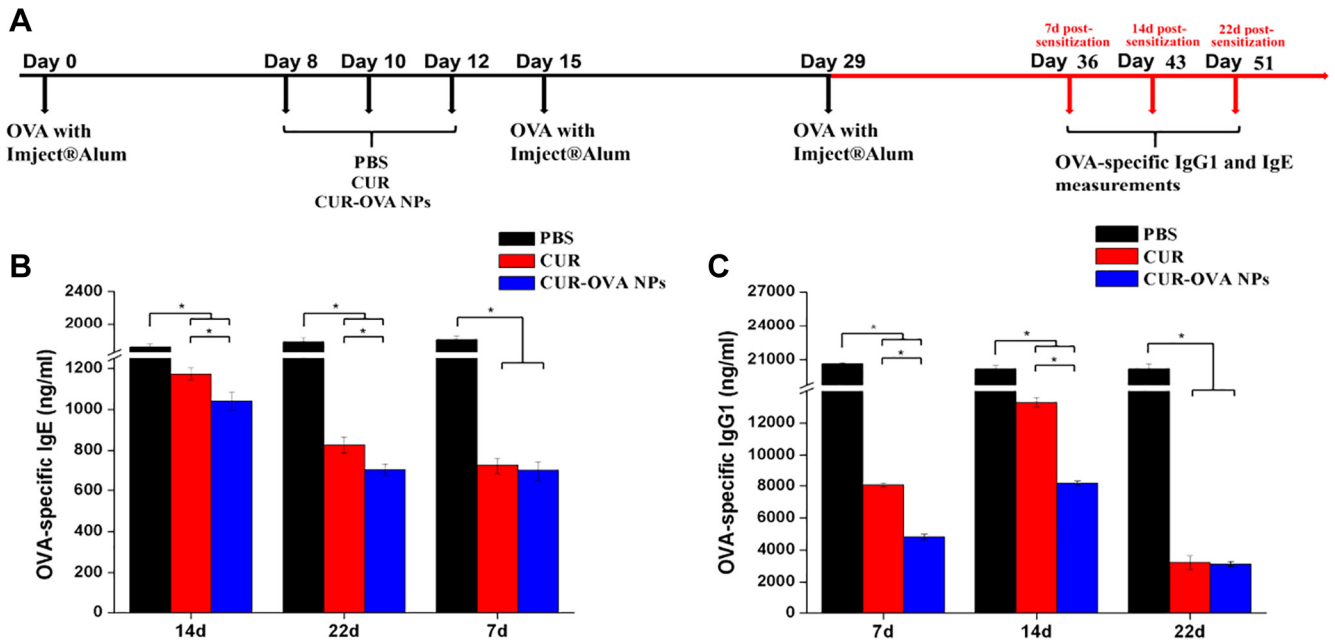


Figure 4. IgE and IgG1 production after treatment with reverse targeting CUR-OVA NPs. (A) Scheme showing the experiment design to measure the amount of OVA-specific IgE and IgG1 in serum after the treatment of CUR-OVA NPs, (B) The amount of OVA-specific IgE on days 7, 14, and 22 after the CUR-OVA NPs treatment, (C) The amount of OVA-specific IgG1 on days 7, 14, and 22 after the CUR-OVA NPs treatment. Each point is presented as the mean $\pm$ SD, $n = 6$ (* $p < 0.05$).

(Figure 4, C) production compared with free curcumin on days 7 and 14 after the last sensitization. The inhibition effect was more remarkable for the IgG1 production. The level of IgG1 in the CUR-OVA NP-treated mice was only 60% of the free-CUR-treated ones on days 7 and 14. These results indicated that CUR-OVA NPs inhibited the sensitization of the mice to the antigen and the curcumin-loaded nanoparticles executed the anti-allergic effect more efficiently owing to the application of OVA NPs. All these data suggested that curcumin and CUR-OVA NPs both exhibit a hyposensitization effect, and the suppressive effect of the CUR-OVA NPs was stronger than that of free curcumin owing to their specific reaction in OVA-sensitized mice.

CD4⁺ T-cell activation assay

The study regarding allergic diseases showed that the production of regulatory T cells could suppress effector T-cell responses,²⁵ and patients with clinical food allergy had a greater number of allergen-specific CD4⁺ T cells.²⁶ These discoveries indicated that CD4⁺ T cells were crucial in modulating an allergic reaction. To explore the relationship between CD4⁺ T cells activation, and the inhibition IgE or IgG1 by CUR-OVA NPs, the CD4⁺ T cells in immunized mouse splenocytes was measured on days 7, 14, and 22 using flow cytometry after the third sensitization (Figure 5, A). As shown in Figure 5, B, the percentage of CD4⁺ T cells significantly decreased in the CUR-OVA NP-treated group compared to the free-curcumin-treated group, with an average percentage of CD4⁺ T cells of 72% in the free curcumin group versus 50% in the CUR-OVA NPs group on day 7. On day 14, the percentages were 70% (free curcumin) and 62% (CUR-OVA NPs). We observed that the inhibitory effect of CUR-OVA NPs to CD4⁺ T cells activation lasted approximately 3 weeks, which was in accordance with the suppression of IgE and

IgG1 antibody production by the injection of CUR-OVA NPs. All these results illustrated that CUR-OVA NPs inhibited CD4⁺ T activity, which would significantly influence the humoral immune response mediated by B cells.

Memory B-cells response assay

To investigate whether CUR-OVA NPs could inhibit memory B cells generation more efficiently, mice treated with CUR-OVA NPs or free curcumin were sacrificed and splenocytes were harvested on days 7, 14, and 22 after the third sensitization, and memory B-cells (CD19⁺ CD27⁺ cells) were detected using flow cytometry (Figure 5, B). As shown in Figure 6, B, the CUR-OVA NPs induced a significant decrease in memory B-cells compared to the free curcumin group. The percentage of CD19⁺ CD27⁺ cells treated with free curcumin was nearly three times of that treated with CUR-OVA NPs on day 14. The decrease in antigen-specific memory B cells was supposed to alleviate the allergy when the body was invaded by the same antigen when inhibiting the immune cells activity.

The targeting of CUR-OVA NPs

By comparing the uptake efficiency of B cells from different antigen-sensitive mice to the CUR-OVA-NPs, the targeting ability can be more successfully affirmed. As shown in Figure 6, the percentage of CD19⁺genipin⁺ B cells was 28% in BSA-sensitized mice, and 42.24% in OVA-sensitized mice. The cellular uptake efficiency of CUR-OVA-NPs was significantly enhanced in OVA-sensitized mice. For the B cells activation, no significant difference was observed in the CD19⁺MHCII⁺ B cells percentage for the free-curcumin-treated group in the BSA- or OVA-sensitized mice (1.24% and 0.80%). However, the

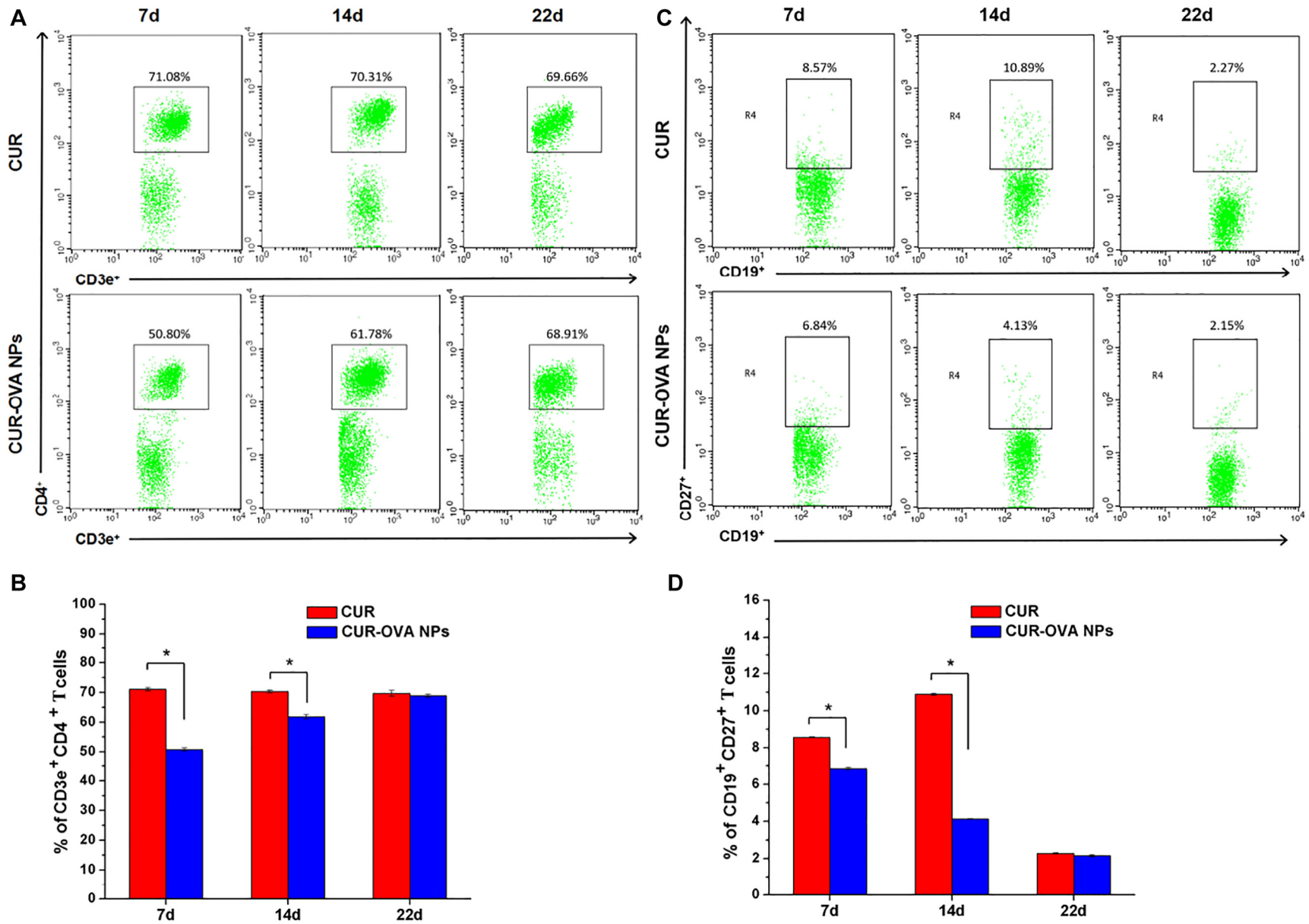


Figure 5. CD4⁺ T-cell activation and memory B-cells response assay. **(A)** Representative flow cytometry scatter diagram of the CD4⁺ T cells in immunized mouse splenocytes treated with CUR and CUR-OVA NPs was measured on day 7, 14 and 22 after the third sensitization, **(B)** Quantification of CD4⁺ T cells treated with CUR and CUR-OVA NPs after the third sensitization. **(C)** Representative flow cytometry scatter diagram of the CD19⁺ CD27⁺ cells in immunized mouse splenocytes treated with CUR and CUR-OVA NPs was measured on day 7, 14, and 22 after the third sensitization, **(D)** Quantification of CD19⁺ CD27⁺ cells treated with CUR and CUR-OVA NPs after the third sensitization. Each point is expressed as the mean $\pm$ SD (n = 6) (**p* < 0.05).

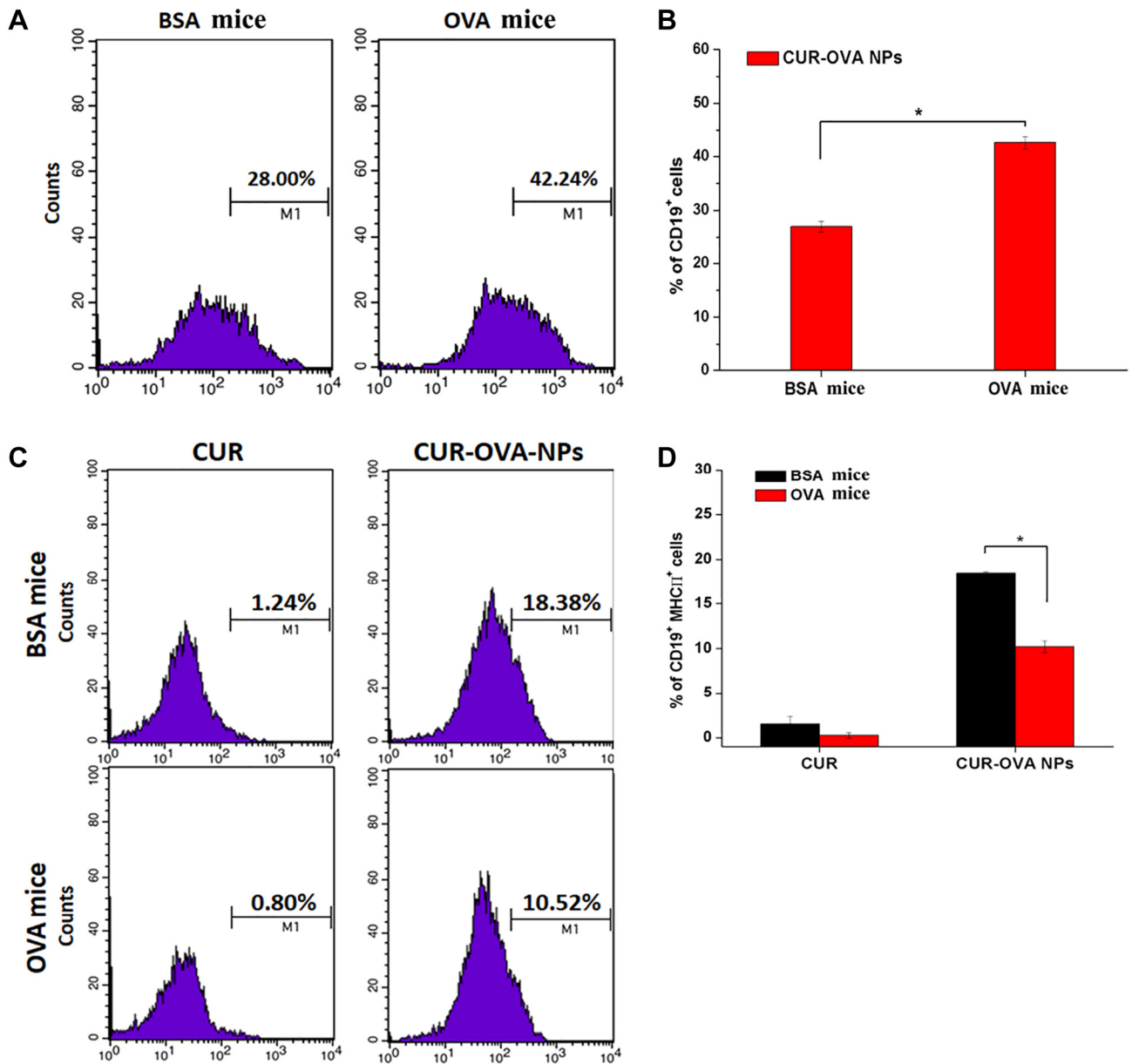


Figure 6. The targeting detection of CUR-OVA NPs by Flow Cytometry. (A) Representative flow cytometry diagram and (B) Quantitative analysis of the proportion of CD19⁺genipin⁺ B cells after treated splenocytes from OVA or BSA-sensitized mice with CUR-OVA-NPs. (C) Representative flow cytometry diagram and (D) Quantitative analysis of the percentage of CD19⁺MHCII⁺ B cells after treated splenocytes from OVA or BSA-sensitized mice with CUR-OVA-NPs or free curcumin. Data were expressed as the mean $\pm$ SD (n = 6) (* p < 0.05).

activation of B cells notably decreased in OVA-sensitized mice (10.52%) compared to the BSA-sensitized ones (18.38%). The CUR-OVA-NPs reliably reduced the activation of B cells in OVA-sensitized mice relative to the BSA-sensitized mice owing to the application of the nanoparticles containing OVA antigens.

Distinguish of multispectral fluorescence in vitro

A multispectral fluorescence imaging in vivo was exerted based on the different fluorescence spectra of two constituents of the CUR-OVA NPs, in which the excitation wavelengths of the

free curcumin and nanoparticles was set as 455 nm and 595 nm, respectively. In view of the special difference for each emission spectrum, the multicolor composite image of the CUR-OVA NPs was obtained with the OVA NP signal (red label) and free curcumin signal (green label). The in vitro multispectral fluorescence images of free curcumin, OVA NPs, and CUR-OVA NPs are shown in Figure 7, A. Without any mutual interference, the OVA NPs (Figure 7, B) and free curcumin (Figure 7, C) signals can be extracted from the CUR-OVA NPs using the multispectral analysis spectrum system. A satisfactory fluorescence resolution can be applied for both the tracking and

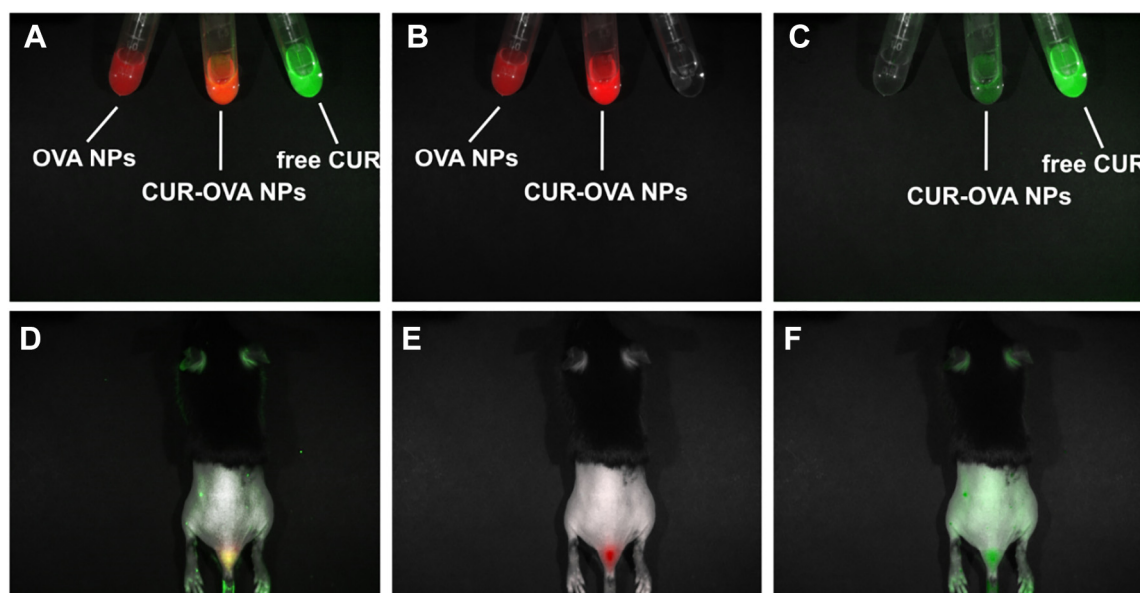


Figure 7. Multispectral fluorescence imaging of dual fluorescence of CUR-OVA NPs. **(A)** Dual fluorescence of OVA-NPs and free OVA RB in vitro, **(B)** Separated fluorescence of OVA-NPs in vitro with red, **(C)** Separated fluorescence of free CUR in vitro with green, **(D)** Dual fluorescence of OVA-NPs and free CUR of CUR-OVA NPs in vivo with yellow, **(E)** Separated fluorescence of the OVA-NPs in vivo with red, **(F)** Separated fluorescence of free CUR in vivo with green.

monitoring of two constituents of the CUR-OVA NPs in vivo. The multispectral fluorescence overlay imaging analysis of CUR-OVA NPs in vivo is shown in Figure 7, D, in which the fluorescence signals of OVA NPs with red (Figure 7, E) and free curcumin with green (Figure 7, F) directly demonstrated the successful separation with multispectral resolution. Therefore, the multispectral fluorescence imaging can clearly distinguish the OVA NPs and free curcumin. Each fluorescent component can monitor the drug delivery to the lymph nodes in real time by a visible imaging system.

In vivo fluorescence tracking of OVA nanoparticles

As shown in Figure 8, A, the representative image directly demonstrated the fate of the OVA nanoparticles and drug metabolism. We found that the fluorescent signal of the CUR-OVA NPs displayed clearly until the 5th day after injection. However, the fluorescence of free curcumin rapidly attenuated, and almost no fluorescent signal could be observed at 6 h after injection. The results proved that the CUR-OVA NPs significantly increased the retention time of curcumin and avoided rapid drug metabolism. Meanwhile, the genipin signal extracted from CUR-OVA NPs and empty OVA NPs showed no obvious difference over time, and the fluorescent signals could still be monitored until day 20. Furthermore, a qualitative comparison of curcumin release and the attenuation of fluorescence from the OVA nanoparticles are illustrated in Figure 8, B. The rapid release of free curcumin is observed clearly, i.e., only 15% of fluorescence remained 3 h after injection. However, for the CUR-OVA NP group, the release rate decelerated; nevertheless, 10% of the fluorescence retained compared to the initial fluorescence intensity on the 14th day. The change in fluorescence intensity of genipin from OVA NPs or CUR-

OVA NPs showed no difference. As visible carriers of drug delivery, OVA NPs could effectively control the release of curcumin, which is paramount for enhancing the anti-allergy ability.

Lymph nodes are important immune organs where both humoral and cellular immunity responses are initiated. The ability of migration toward lymph nodes is essential for antigens to exert their functions in vivo. In Figure 8, C, the fluorescence of CUR-OVA NPs, OVA-NPs, and free curcumin in the draining lymph nodes demonstrated the migration from the injection sites within 12 h. No visible fluorescence was found in the lymph node for free-curcumin-treated mice, thus illustrating that the free drugs could not easily deliver to targeted immune organs. However, for the OVA-NPs or CUR-OVA NPs, obvious fluorescence appeared in the lymph node 3 h after injection. The results indicated that nanoparticles can rapidly migrate to the draining lymph node, thus suggesting that the loaded drug of CUR-OVA NPs could be delivered to the lymph nodes. As expected, the fluorescence of curcumin loaded on the CUR-OVA NPs appeared in the draining lymph node at 3 h. However, owing to the excitation wavelength of curcumin at 455 nm, the signal was affected by the autofluorescence of skin and was relatively weak. In general, the in vivo tracking images suggested that the CUR-OVA NPs could carry the drugs and migrate to the draining lymph node efficiently.

Discussion

Many studies illustrated that curcumin can exhibit pharmacologic activities such as anti-inflammatory, anticancer, and anti-oxidant effects. It can even attenuate the development of hypersensitivity diseases including asthma or allergic conjunctivitis as an allergic

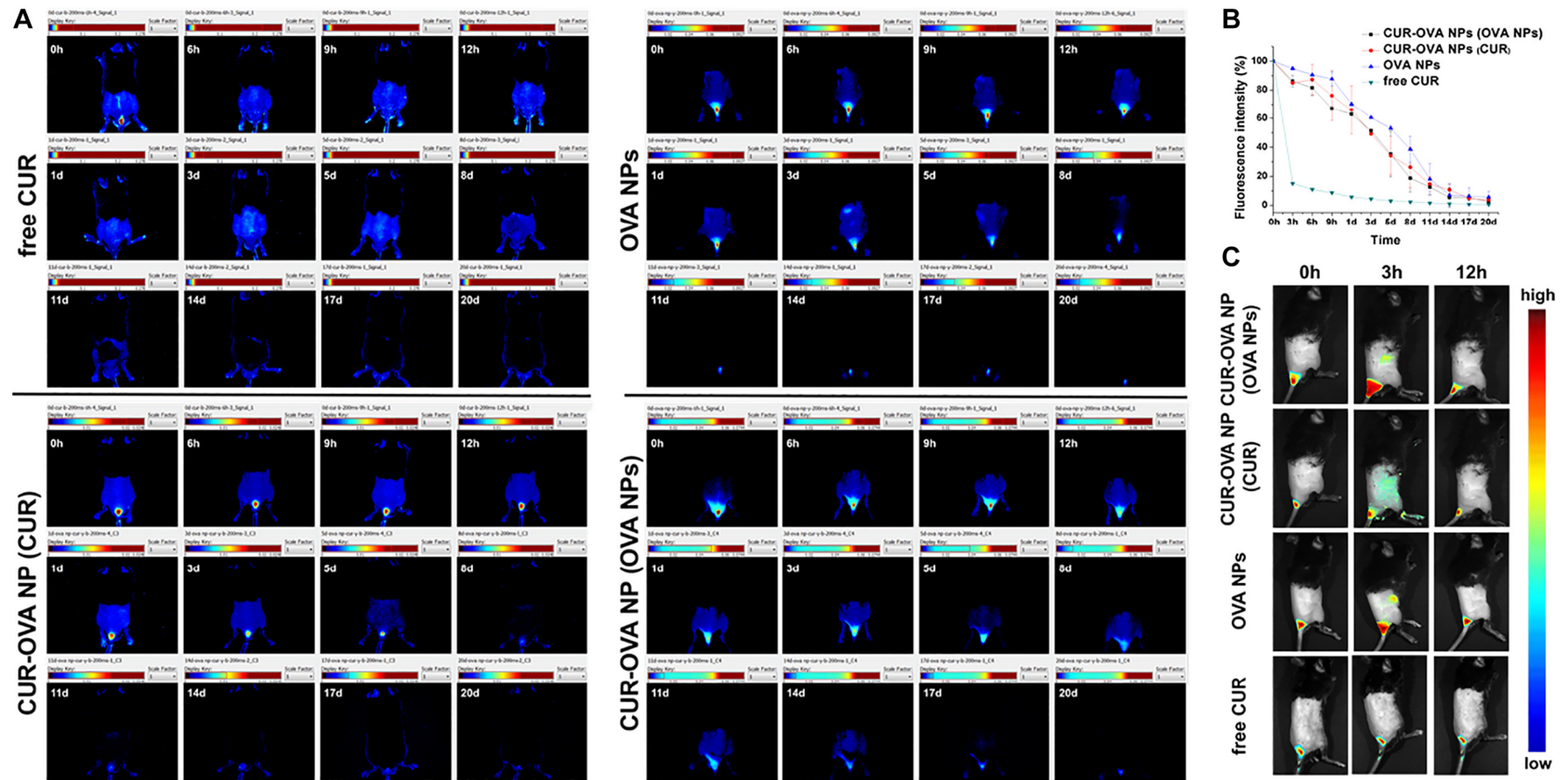


Figure 8. In vivo fluorescence tracking of free CUR, OVA-NPs and CUR-OVA NPs in the injection site and draining lymph nodes based on the fluorescent signal of OVA-NPs and CUR. **(A)** Representative fluorescence imaging in vivo for free CUR, OVA-NPs and CUR-OVA NPs with rainbow color ($n = 3$), **(B)** Quantitative analysis of fluorescent intensity of free CUR, OVA-NPs and CUR-OVA NPs by the Maestro software. Fluorescent signal at the injection site expressed as a percentage of initial signal. Data were expressed as the mean $\pm$ SD ($n = 3$), **(C)** Nanoparticles migrated into draining lymph nodes from the injection sites within 12 h with rainbow color.

immunosuppressor. However, unfavorable characteristics limit its bioavailability such as low aqueous solubility, rapid metabolism, low gastrointestinal absorption, and degradation at alkaline pH. Therefore, improving the efficiency and clinical effect of curcumin would be a potential approach using a drug carrier system for sustained release and targeted delivery. Wenrui et al. formulated curcumin-solid lipid nanoparticles (curcumin-SLNs) and proved that the administration of curcumin-SLNs into OVA-induced asthmatic rats could significantly reduce the airway hyper-responsiveness and the number of inflammatory cells in lung tissue compared to the curcumin-treated group.¹¹ A similar study reported that PEGylated curcumin NPs exhibited excellent dispersibility and outstanding stability in physiological environments and the therapeutic efficacy was considerably better than that of free curcumin.²⁷ To improve the therapeutic effect further, the functional modification of NPs by targeting molecules is an important strategy. A successful targeting implies sufficient stability to avoid premature drug release into the bloodstream prior to the target arrival.

Targeting to tissues and cells can typically be achieved by the passive or active mode. The current research primarily focuses on the active targeted therapy that is achieved by the modification of a drug delivery system (DDS) with receptor-specific ligands.²⁸ Unlike the conventional targeting DDS, the reverse targeting drug delivery system (RT-DDS) attracted our attention as an innovative targeting drug delivery system. Kanae Ichikawa proposed an entirely new type of RT-DDS, where antigen-modified liposomes (OVA-lipo) were used as a drug carrier targeting the immune cells through the specific recognition of antigens.¹⁴ The study illustrated that OVA-lipo DOX could suppress the production of OVA-specific IgE and IgG antibodies in OVA-sensitized mice for a long term, suggesting a new treatment strategy for immune diseases such as allergy. Inspired by this idea, we formulated CUR-OVA NPs to demonstrate the favorable dual functions of targeting and delivery by the simple self-crosslinking pattern without any addition of other carrier materials. Our previous report has verified that OVA NPs were more efficient in DC intake and promoting DC maturation compared with soluble OVA²³; therefore, OVA NPs would be more efficiently recognized by immune cells than OVA-modified NPs. Additionally, OVA NPs also served as natural carriers for immunosuppressive drug curcumin. By the sustained release of curcumin, CUR-OVA NPs could inhibit the activation of the targeted immune cells, and would thus provide a valid treatment for immune diseases such as allergy. In addition, based on the dual fluorescence of the genipin crosslinked carrier and curcumin, a visible reverse targeting delivery system for CUR-OVA NPs successfully merged the function of targeting, vehicular, and fluorescence.

Initially, we confirmed that OVA NPs could be better recognized than free OVA in OVA-sensitized mice, which cause plasma cells to produce more IgG1 and IgE antibodies (Figure 3). This result suggested that if OVA NPs were used as a delivery system, the drugs might be delivered to sensitized cells such as T cells, B cells, mast cells, or other related immune cells. That is to say, OVA NPs could be applied not only as drug carriers, but also as target molecules.

To prove the dual function of OVA NPs, curcumin was encapsulated into the NPs as an anti-allergy drug. Once CUR-

OVA NPs were injected into the OVA-sensitized mice, the mice were exposed to the same antigen again and the immune cells would produce a series of responses. Specific B cells will capture the antigen to be activated and become the antibody-producing plasma cells or a small proportion of memory B cells. In addition, allergen exposure and subsequent cellular events will lead to the synthesis and release of IgE, further trigger mast cells degranulation, and subsequently cause the release of histamine, leukotrienes, tryptase, or cytokines (IL-4, IL-5, and TNF- α , etc.).²⁹ Consequently, curcumin encapsulated in OVA NPs could inhibit the activation of these immune cells through the recognition of immune cells to OVA. Previous reports have revealed that curcumin could inhibit the IgE-mediated degranulation of mast cells.³⁰ Herein, other related immune cells response was measured including CD4⁺ T cells and memory B cells. Figure 5 demonstrates that the CUR-OVA NPs directly reduced the percentage of CD4⁺ T cells in splenocytes, which was one of the key factors that triggered the allergic inflammatory reaction. Many studies reported that the effector CD4⁺ T cell expansion was necessary during sensitization^{31,32} and was thought to be the dominant active T cell subtype in clinical allergic asthma as well as in the animal models of asthma.³³ Our results indicated that CUR-OVA NPs might inhibit helper T-lymphocytes, which directly influenced the maturation and differentiation of B cells as well as the subsequent antibody production.

Once the body is exposed to the same antigen, memory B cells will rapidly proliferate and differentiate into plasma cells to mediate the recalling immunity response.³⁴ Therefore, the recurrence of allergy is closely related with the production of memory B cells.³⁵ The results of the present study indicated that the percentage of memory B cells significantly decreased in the mice treated with CUR-OVA NPs, which would significantly reduce the risk of allergy. Many previous studies have reported that curcumin possessed anti-allergic activity in animal models including allergic conjunctivitis or asthma, and that curcumin NPs could increase the bioavailability of drugs and avoid rapid metabolism.^{12,13,36} However, the influence on antigen-specific memory B cells has rarely been studied, and the long-term impact on anti-allergy is still unknown. Herein, OVA NPs as a curcumin carrier significantly increased the opportunity of contacting OVA-sensitized immune cells, which not only directly decreased the production of IgG1 and IgE antibodies but also inhibited the generation of memory B cells. However, for a long-term anti-allergic effect, many important factors should be considered including the administration route, drugs-loaded pattern, or administration schedule. By optimizing these aspects in accordance with the type of allergic diseases, the effect might be further improved.

The targeting can be better affirmed by comparing the uptake efficiency of B cells from the different antigen-sensitized mice to the CUR-OVA-NPs. The results indicated that the cellular uptake of the NPs significantly enhanced for the OVA-specific B cells. In addition, to verify that the suppressive effect of the putatively targeting NPs was specific in contrast to free curcumin further, the activation of B cells from different antigen-sensitized mice was measured after treatment with CUR-OVA-NPs or free curcumin. The activation of B cells notably decreased in OVA-

sensitized mice compared to the BSA-sensitized ones. Although the mechanism is not well known, the CUR-OVA-NPs reliably reduced the activation of B cells in OVA-sensitized mice owing to the application of the NPs containing OVA antigens. In addition, we also found the percentage of CD19⁺MHCII⁺ B cells significantly increased in the CUR-OVA-NPs treated group compared to that in the free-curcumin-treated ones for both OVA- and BSA-sensitized mice. It was supposed that free curcumin cannot activate B cells, but the BCR on the B cells surface can recognize the OVA antigen from the CUR-OVA-NPs, which further improved the expression of MHCII and stimulated B cell activation. The result suggested that the hyposensitization effect of curcumin was not caused by the direct damage to immune cells, but by modulating other signaling molecules such as inhibiting the MAPK/NF- κ B pathway.³⁰ In summary, we verified that the suppressive effect of the putatively targeting NPs was specific in contrast to free curcumin using different antigen-sensitized mice models.

Meanwhile, the fluorescence imaging of OVA NPs is an advanced skill for noninvasively monitoring the drug delivery and the antigen presentation system. In this study, a dual fluorescent system was studied with CUR-OVA NPs based on the fluorescence signals of curcumin and genipin crosslinked OVA NPs. More importantly, the multispectral fluorescence imaging method can successfully separate the fluorescence signals of curcumin and OVA NPs. Moreover, fluorescence intensities can reveal the drug delivery process. The in vivo imaging results suggested that CUR-OVA NPs can deliver the drugs and migrate to the draining lymph nodes efficiently. The combination of targeting OVA NPs and fluorescence imaging yielded a novel visible drug delivery system for allergy treatment.

In summary, a dual fluorescent reverse targeting drug delivery system based on CUR-OVA NPs was successfully developed for allergy treatment, which was recognized by antigen-specific immune cells and suppressed antigen-specific IgG1 and IgE production. The reverse targeting drug delivery system of CUR-OVA NPs suppressed OVA-induced allergy, exerting a stronger and more effective treatment on the immunomodulatory role. The fluorescence intensities revealed the precise process of the drug and carrier degradation, and recorded the matured DCs migrating to the lymph nodes for the effective allergy immunotherapy. A dual fluorescent reverse targeting delivery system based on CUR-OVA NPs successfully merged the functions of targeting, vehicles and fluorescence, which provided an innovative strategy for visible allergy treatment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nano.2018.11.010>.

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A dual fluorescent reverse targeting drug delivery system based on curcumin-loaded ovalbumin nanoparticles for allergy treatment

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Supplementary Material

Materials

Ovalbumin (OVA, from chicken egg white, lyophilized powder, $\geq 98\%$) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorochrome-conjugated anti-mouse antibody obtained from Biolegend (San Diego, CA, USA): cy5.5-anti-CD3e, cy5.5-anti-CD19, APC-anti-CD27 and APC-anti-MHCII. FITC-anti-mouse CD4 antibody were obtained from eBioscience (San Diego, CA, USA). Anti-Ovalbumin IgG1 and IgE Mouse ELISA kits were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Genipin (98%) was purchased from Xi'an Plant Bio-engineering Corporation (Xi'an, China). Polyethyleneimine (PEI, $M_w=10000$) was provided by Aladdin Chemistry Corporation (Shanghai, China). Curcumin ($>98\%$ purity) was obtained from Acros Organics (Morris Plains, NJ, USA). Imject® Alum was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade.

Cells and mice

BMDCs was prepared according to our previous report[1]. Six-week-old female BALB/c mice and C57BL/6 mice were housed with free access to food and water. All animal procedures were conducted following the protocol approved by the Institutional Laboratory Animal Ethics Committee and the Institutional Animal Care and Use Committee (IACUC), Peking Union Medical College, People's Republic of China. All animal experiments were used in accordance with approved institutional protocols established by Peking Union Medical College, People's Republic of China.

Preparation and characterization of OVA NPs and CUR-OVA NPs

OVA-genipin-PEI nanoparticles (OVA NPs) were prepared by genipin cross-linked reaction between OVA and PEI. Briefly, genipin solution (1% w/v, 643 μ L) was added to the OVA solution (5mg/mL, 1.5 mL) to stir over night and then PEI solution (15mg/mL, 1 mL) was added with stirring for 48 h. Afterward, the system was dialyzed (MWCO = 3500Da) against de-ionized water for 24 h to remove excess genipin and were lyophilized in a tabletop lyophilizer.

The curcumin loaded OVA NPs (CUR-OVA NPs) were further prepared by the mixed solvent method[2]. OVA NPs were swelled by the mixed DMSO/water solution, and then curcumin was

encapsulated into OVA NPs by the diffusion of the dissolved curcumin with stirring for 24h. After that, the solution was dialyzed (MWCO 3500Da) against de-ionized water for 24h and lyophilized to obtain CUR-OVA NPs. The encapsulation efficiency (EE) and drug loading rate (DL) were calculated by the using following equation: $EE (\%) = \text{weight of curcumin retained in nanoparticles} / \text{weight of the initial curcumin} \times 100$; $DL (\%) = \text{weight of curcumin retained in nanoparticles} / \text{weight of the curcumin loaded in OVA nanoparticles} \times 100$.

Spatial structure characterization of OVA and OVA NPs were performed by Circular Dichroism Chiroptical Spectrometer (J-815 CD spectrometer, JASCO, JAPAN). The morphology of the prepared nanoparticle was characterized by atomic force microscopy (AFM) (Multi Mode8, Veeco Instruments, USA). The mean size, size distribution and zeta potential of the OVA NPs and CUR-OVA NPs were determined with a NY 90-Plus particle size analyzer and zeta potential analyzer (Nano ZS, Malvern Instruments, UK). UV-Vis and fluorescence spectra were measured by a multimode microplate spectrum photometer (Varioskan™ Flash, ThermoFisher Scientific, USA).

In vitro release of curcumin

The in vitro drug release were evaluated by using a neutral and acidic phosphate saline buffer with pH of 7.4 and 5.5 containing 0.5% (w/w) tween 80, respectively. A known amount of curcumin loaded OVA nanoparticles were dialyzed (MWCO=3500) against 10 mL phosphate buffer solution. The system was incubated at a shaker in 37 °C with the speed of 150 rpm. At definite time intervals, the released curcumin was determined by HPLC (Waters e2695, USA) at 430 nm. During the detections, the same volume of samples were taken out and then the fresh buffer solution was added. The cumulative release ratio of the curcumin was calculated using the standard curve method.

Cellular toxicity of CUR-OVA NPs

The cellular toxicity of free OVA, free curcumin, OVA NPs and CUR-OVA-NPs was measured by WST-1 assay. Briefly, BMDCs were seeded into 96-well culture plates (1×10^4 cells/well) in growth medium for 2h, then the cells were incubated with different concentrations of free OVA, curcumin or nanoparticles. After incubating for 24h, 20 μ L of WST-1 solution was added to each well and incubated for 4 h at 37°C, OD450 was measured using a Multifunction Microplate Reader (Varioskan™ Flash; Thermo Fisher Scientific, USA).

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