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ORIGINAL RESEARCH

Chimeric Antigen-LgDNA Nanoparticles Attenuate Airway Th2 Polarization

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Introduction: The therapeutic efficacy for airway allergies needs to be improved. Th2 polarization is a primary pathological feature of airway allergies. We constructed chimeric antigen-LgDNA (*Lactobacillus rhamnosus* DNA) nanoparticles (CAP-NPs). The effects of CAP-NPs on reconciling airway Th2 polarization were tested.

Methods: In this study, disulfide bond-linked antigen-major histocompatibility complex II (MHC II)-LgDNA nanoparticles (NPs) were constructed and designated CAP-NPs. An airway Th2 polarization mouse model was established to test the effects of CAP-NPs on suppressing the Th2 response.

Results: The CAP-NP components of ovalbumin (OVA), major histocompatibility complex II (MHC II), and LgDNA were confirmed in a series of laboratory tests. The CAP-NPs remained stable at pH7.2 for at least 96 h. In in vitro experiments, CAP-NPs bound to the surface of OVA-specific $CD4^+$ T cells, which resulted in apoptosis of the antigen-specific $CD4^+$ T cells. Removal of any of the three components from the NPs abolished the induction of apoptosis of antigen specific $CD4^+$ T cells. CAP-NPs increased the expression of lysine-specific demethylase 5A (KDM5A) in $CD4^+$ T cells. Histone H3K9 and the gene promoter of caspase 8 were demethylated by KDM5A, which led to transcription and expression of the caspase 8 gene. Administration of CAP-NPs significantly alleviated experimental airway Th2 polarization through activating the caspase 8-apoptosis signaling pathway.

Discussion: In this paper, we constructed CAP-NPs that could induce antigen-specific $CD4^+$ T cell apoptosis. Administration of CAP-NPs efficiently alleviated experimental airway Th2 polarization.

Keywords: airway allergy, T cell, therapy, vaccine, nanoparticle

Introduction

Th2 polarization is a crucial factor associating with the pathogenesis of numerous immune disorders, such as allergic rhinitis, allergic asthma, allergic dermatitis, and certain autoimmune diseases.[1,2] Th2 polarization indicates that a large number of Th2 cells aggregate in the local tissues. Th2 cells produce higher than the required quantities of Th2 cytokines.[3] Th2 cytokines promote IgE production in plasma cells. Binding of IgE to the high-affinity IgE receptor (FcεRI) sensitizes mast cells. The release of allergic mediators, which triggers allergic attacks, occurs when sensitized mast cells are re-exposed to specific antigens.[4] The treatment of Th2 polarization-related diseases is not satisfactory currently. Most therapies are for the symptom control, such as anti-histamines and steroids.[5] Although the allergen specific immunotherapy is an etiology targeting remedy, its efficacy needs to be further improved.[6] Thus, there are only limited satisfactory therapies available for Th2 polarization currently.[7] Therefore, it is urgent to develop better therapies for Th2 polarization-related diseases.

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Referring to a published strategy,[8] we constructed a new type of nanoparticle containing the specific antigen ovalbumin (OVA), major histocompatibility complex II (MHC II), and LgDNA (DNA extracted from the probiotic strain *Lactobacillus rhamnosus GG*). Specifically, this nanoparticle (NP) only has a peptide carrier to which the three substances are connected by disulfide bonds. This was designated as CAP-NP. Administration of CAP-NPs via nasal instillations effectively suppressed experimental Th2 polarization in the airways.

Materials and Methods

Reagents

Antibodies () against H3K9 (Cat#ab8898) and H3 (ab1791) were purchased from Abcam (Cambridge, UK). Antibodies against CD4 (sc-19641; fluorochrome: AF488), CD11c (sc-1185, AF546), CD19 (sc-390244, AF594), F4/80 (sc-52664, AF648), IL-4 (sc-32242, AF700), CD44 (sc-53068, AF790), OVA (sc-65984), and MHC II (sc-59318) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IETD-CHO was purchased from Sigma–Aldrich (St. Louis, MO, USA). MHC II was obtained from Abcam. MHC II enzyme-linked immunosorbent assay (ELISA) kit was purchased from Jianglai Biotech (Shanghai, China). OVA323-339 peptide, Annexin V kit, propidium iodide, and FITC-labeling kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). The information for ELISA kits includes EPX (Kemiao Biotech, Wenzhou, China), Ki-67 (Kemiao Biotech, Wenzhou, China), Mcpt1 (MultiSciences, Hangzhou, China), IL-4 (MultiSciences, Hangzhou, China), IL-5 (MultiSciences, Hangzhou, China), IL-13 (MultiSciences, Hangzhou, China). OVA-specific IgE (Yuntai Biotech, Beijing, China).

Mice

Male BALB/c mice (6-8-week-old) were used in this study to prevent potential effects on menstruation. Mice were acquired from the Guangdong Experimental Animal Center in Fushan, China. DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed at the Shenzhen University Animal Center, a pathogen-free facility (Facility grade: SPF. Facility certificate number: SYXK-2022-0302. Mouse grade: SPF. Mouse certificate number: No.44007200117006). Water and food were provided by the mice. Approval#2023028 for the animal experimental protocol was obtained from the Animal Ethics Committee of Shenzhen University. The animal experiments were performed in accordance with the guidelines about experimental animal care and use in China.

Preparation of a Peptide Carrier

The sequence of the peptide carrier was adopted from a report by Wang et al,[9] with some modifications. The sequence is "Cys-Trp-Pro-Arg8-Pro-Arg8-Pro-Arg8-Cys" was provided by Sangon Biotech (Shanghai, China).

Preparation of the CAP-NPs (OVA Peptide-MHC II-LgDNA Nanocomposites)

Following a published strategy,[9] CAP-NPs were prepared by mixing 200 μg/mL of the peptide carrier solution with 100 μg/mL of $OVA_{323-339}$ solution, 100 μg/mL of MHC II protein, and 50 μg LgDNA. The mixture was incubated for 2 h at room temperature to allow disulfide cross-linking. The mixture was then subjected to an increase in temperature from 4°C to 95°C and then returned to 4°C. The samples were vibrated at 300 rpm for 5 min. The diameter and size distribution of the CAP-NPs were determined by dynamic light scattering (DLS), based on published procedures.[10]

Scanning Electron Microscopy

CAP-NP samples were coated with Pd-Au, observed, and photographed using a scanning electron microscope (TESCAN, Czech Republic).

Assessment of the Stable Property of CAP-NPs

CAP-NPs were diluted to 1 μg/mL in PBS (pH7.2) at room temperature. The samples were collected at 24, 48, 72, and 96 h. To assess if all three components, OVA, MHC II, and LgDNA, remained stable in the NPs, cross-ELISA and qPCR were employed.

Enzyme-Linked Immunosorbent Assay (ELISA)

Proteins in the samples were quantified using ELISA with commercial kits following the protocols provided by the manufacturers.

Crossing-ELISA

Microplates were coated with OVA Ab (1 μg/mL) or MHC II Ab (1 μg/mL) overnight. NP samples (1 μg/mL, 0.5 mL) were added to the wells (in triplicate) and incubated at 4°C overnight. After washing three times with PBST (PBS containing 0.05% Tween 20), MHC II Ab was added to the OVA Ab-coated plates and OVA Ab was added to the MHC II-coated plates. Bovine serum albumin (BSA) was added to the negative control wells instead of samples. The plates were incubated for 2 h at room temperature. After washing, biotinylated anti-mouse Fab antibody was added to each well. Two hours later, horseradish peroxidase-streptavidin was added to each well. TMB was added to the wells and the reaction was stopped by adding 2 M H_2SO_4. The absorbance of the plates was measured at 450 nm wavelength using a microplate reader (HBS1101).

Quantification of LgDNA Using qPCR

Samples were collected to assess the stability of CAP-NPs and analyzed by qPCR using a SYBR Green Master Mix kit in the presence of a pair of LGG primers (tgagctacggttgatgttgc and tggtggaggtgaggagaatc). The results were calculated using the $2^{-\Delta\Delta Ct}$ method and are presented as relative expression (RE) against the gene encoding glyceraldehyde-3-phosphate dehydrogenase A *gapA* (; aggtctgatgaccaccgttc and ggaacgccataccagtcagt).

Assessment of CAP-NP Binding to Antigen Specific $CD4^+$ T Cells

$CD4^+$ T cells were isolated from the spleens of DO11.10 mice and BALB/c mice by flow cytometry. The cells were then exposed to FITC-CAP-NPs (1 μg/mL) for 2 h. The cells were then stained with propidium iodide (PI) to determine cell morphology and observed using confocal microscopy. In contrast, DO11.10 mice and BALB/c mice received nasal instillations (20 μL/nostril; containing FITC-CAP-NPs 1 mg/mL). Three hours later, single cells were prepared from the airway tissues of the mice and analyzed using flow cytometry.

Preparation of Single Cells with the Airway Tissues

The lungs were excised after sacrifice and cut into small pieces. Collagenase IV (0.5 mg/mL) was added and incubated at 37 °C for 30 min with mild agitation. Single cells were filtered through a cell strainer and used in other experiments.

Cell Culture

Immune cells were cultured in RPMI1640 medium, which was supplemented with 10% fetal calf serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mm l-glutamine. Trypan blue exclusion assay was used to assess cell viability, which exceeded 99%.

Flow Cytometry

The cells were stained with fluorescence-labeled Abs (Abs are detailed in the figures) or isotype IgG for 30 min at 4°C. To stain intracellular molecules, cells were fixed with 1% paraformaldehyde (containing 0.05% Triton X-100) for 1 h. The cells were then stained with fluorescence-labeled antibodies. After washing with PBS, the cells were analyzed using a flow cytometer (BD FACSCanto II). The results were processed using the (FlowJo). Isotype IgG staining data were used as gating references.

Isolation of $CD4^+$ T Cells

Single cells were prepared from lung tissues, as described above. $CD4^+$ T cells were isolated from single cells by flow cytometry. The purity of isolated $CD4^+$ T cells was determined using flow cytometry. If the purity did not exceed 90%, isolation was repeated.

RNA Sequencing

TRIzol reagent was used to extract RNA from $CD4^+$ T cells. The samples were then sent to a biotech company (BGI; Shenzhen, China). The technical staff of the company conducted RNA sequencing of the RNA samples. The data were also analyzed by the professional staff of the company. Significant attention was paid to identifying differentially expressed genes (DEGs) in $CD4^+$ T cells, which are presented as heat maps.

Real-Time Quantitative RT-PCR (RT-qPCR)

TRIzol reagent was used to extract RNA from the cells. In accordance with the manufacturer's protocol, a reverse transcription kit was used to convert the cDNA from the RNA samples. The cDNA samples were amplified using a qPCR device (CFX96, Bio-Rad) and a SYBR Green Kit. Primers used in this study included *Kdm5a* (cctggcagtaggagcaaaag and cgaccacaaaacatgcaaac), *Casp8* (ggcctccatctatgacctga and tgtggttctgttgctcgaag), *Fas* (tgtgaacatggaacccttga and ttcagggtcatcctgtctcc), and *Fasl* (catcacaaccactcccactg and gttctgccagttccttctgc). The formula $2^{-\Delta\Delta Ct}$ was employed to calculate the results and is presented as relative expression (RE) against the housekeeping gene *Actb* (agccatgtacgtagccatcc and ctctcagctgtggtggtgaa).

Chromatin Immunoprecipitation (ChIP)

$CD4^+$ T cells were fixed in 1% formalin for 15 min. The cells were lysed using lysis buffer. DNA was sheared into small pieces by sonication of lysates. Protein G agarose beads were used to pre-clear pre-existing immune complexes in the samples. The beads were then discarded. The samples were incubated overnight with the antibodies of interest. Beads were harvested by centrifugation. An eluting buffer was used to elute the samples on beads. DNA was extracted from the samples using a DNA recovery kit (Qiagen) and analyzed by qPCR in the presence of a pair of primers for the *Casp8* gene promoter (ccatcaggcaggcctacagc and ggcctaggtcagatccatca). The results are presented as fold changes relative to the input. Proteins in the samples were quantified using ELISA with specific reagent kits following the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

Commercial kits were used to quantify the proteins in the samples, according to the manufacturer's protocols.

Establishment of an Airway Allergy Mouse Model

Randomly grouped mice (6 mice per group) were sensitized to ovalbumin (OVA) by subcutaneous injection with OVA (0.1 mg OVA/mouse mixed in 0.1 mL alum) on the back skin on day 1 and day 7, respectively. Mice were boosted with nasal instillations (20 μL/nostril containing 5 mg OVA/mL) daily from day 9 to day 22. On day 23, mice were challenged with a large dose of OVA (20 μL/nostril containing 50 mg OVA/mL). Two hours after the challenge, mice were sacrificed by cervical dislocation. The trachea was exposed on the neck immediately after the sacrifice. One milliliter of saline was introduced into the lungs using a syringe. The saline was recovered immediately using the same syringe. The lavage procedures were repeated two more times. The lavage fluids of three times were pooled, and used as bronchoalveolar lavage fluids (BALF) in other experiments.

Statistics

The Student's *t*-test was used to determine the difference between the data collected from the two groups. Analysis of variance (ANOVA) followed by the Bonferroni test or Dunnett's test was used to analyze data from multiple groups. The correlation between data from the two groups was evaluated using Pearson's correlation coefficient test. Statistical significance was set at $p < 0.05$. The statistical results are presented as $*p<0.05$; $**p<0.01$; $***p<0.001$; and $****p<0.0001$.

Results

Characteristics of CAP-NPs

The CAP-NPs were observed using scanning electron microscopy. The NPs were approximately 50–100 nm (Figure 1A). CAP-NPs were tested by diluting with PBS, SDS, or urea. The samples were measured using the dynamic light-scattering

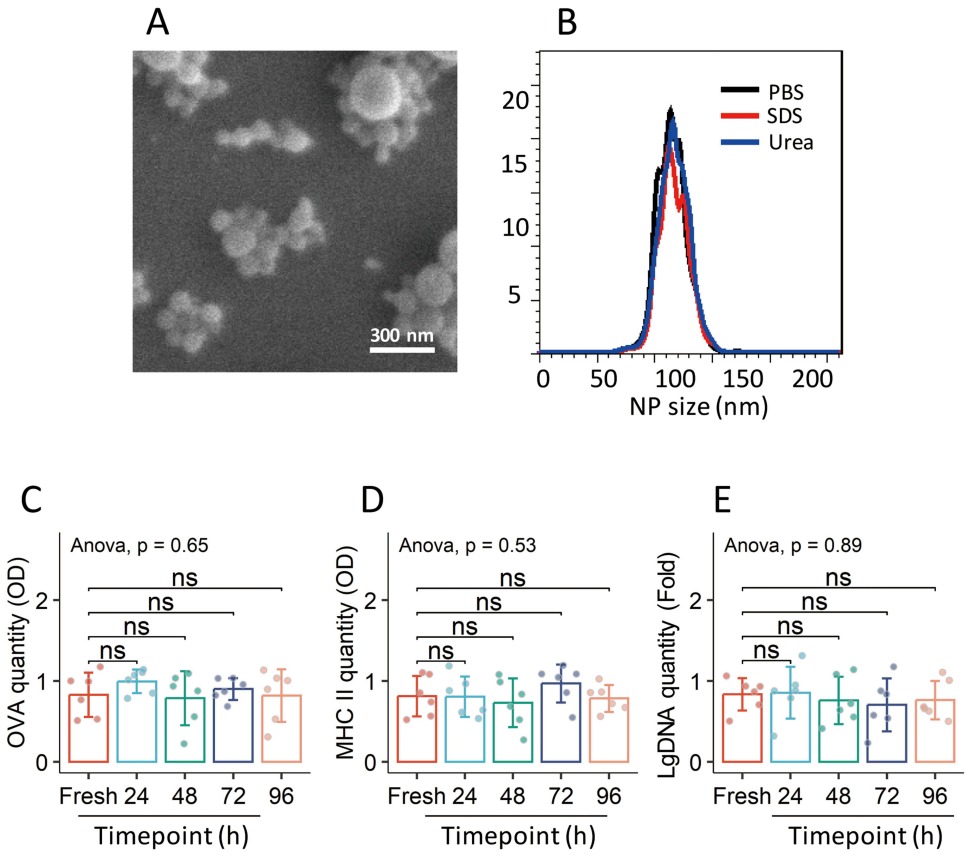

Figure 1 Characteristics of CAP-NPs. (**A**) A representative SEM image of CAP-NPs (original magnification: ×20K). (**B**) CAP-NPs were treated with PBS, or SDS (0.05 mm), or urea (0.06 mm). NP size was then measured by dynamic light scattering method. The curves show size of disulfide NPs. (**C**–**E**), CAP-NPs were diluted with PBS (1 μg/mL). Samples were harvested at indicated timepoints, and quantified by cross-ELISA or qPCR. Bars indicate the quantity of indicated molecules. The data are presented as mean ± SD. Each dot in bars presents one sample. Statistics: ANOVA + Dunnett's test.
Abbreviation: ns, Not significant.

method. The results showed that the particle size was distributed in a range of 50–100 nm. The distribution curves of the three methods overlapped (Figure 1B). We then tested the stability of the CAP-NPs. PBS (pH 7.2) was added to the CAP-NPs (1 μg/mL) and the solution was incubated at 37°C. Samples were harvested from CAP-NP-containing PBS at 24, 48, 72, and 96 h and assessed using cross-ELISA. The results showed that the quantities of the three components, OVA, MHC II, and LgDNA, did not change significantly between the four time points when compared with the freshly prepared CAP-NP samples (Figure 1C–E). The results indicate that the three components of CAP-NPs do not separate or remain stable in a pH7.2 environment at least for 96 h.

CAP-NPs Bind to Antigen Specific $CD4^+$ T Cells

$CD4^+$ T cells were isolated from the spleens of DO11.10 mice or BALB/c mice and cultured. FITC-labeled CAP-NPs were added to the culture and incubated for 1 h. The cells were harvested and processed for confocal microscopy. We observed that CAP-NPs bound to the surface of DO11.10 $CD4^+$ T cells but not BALB/c $CD4^+$ T cells (Figure 2A and B). We then treated DO11.10 mice or BALB/c mice with nasal instillation (containing CAP-NPs). Mice were sacrificed 3 h later. Single cells were prepared from airway tissues and analyzed using flow cytometry. We found that more than 80% of the CAP-NP-bonded cells were DO11.10 $CD4^+$ T cells. Less than 3% of the CAP-NP-bound cells were DCs, macrophages, B cells, or BALB/c $CD4^+$ T cells (Figure 2C–H). These results demonstrate that CAP-NPs can specifically bind to antigen-specific $CD4^+$ T cells.

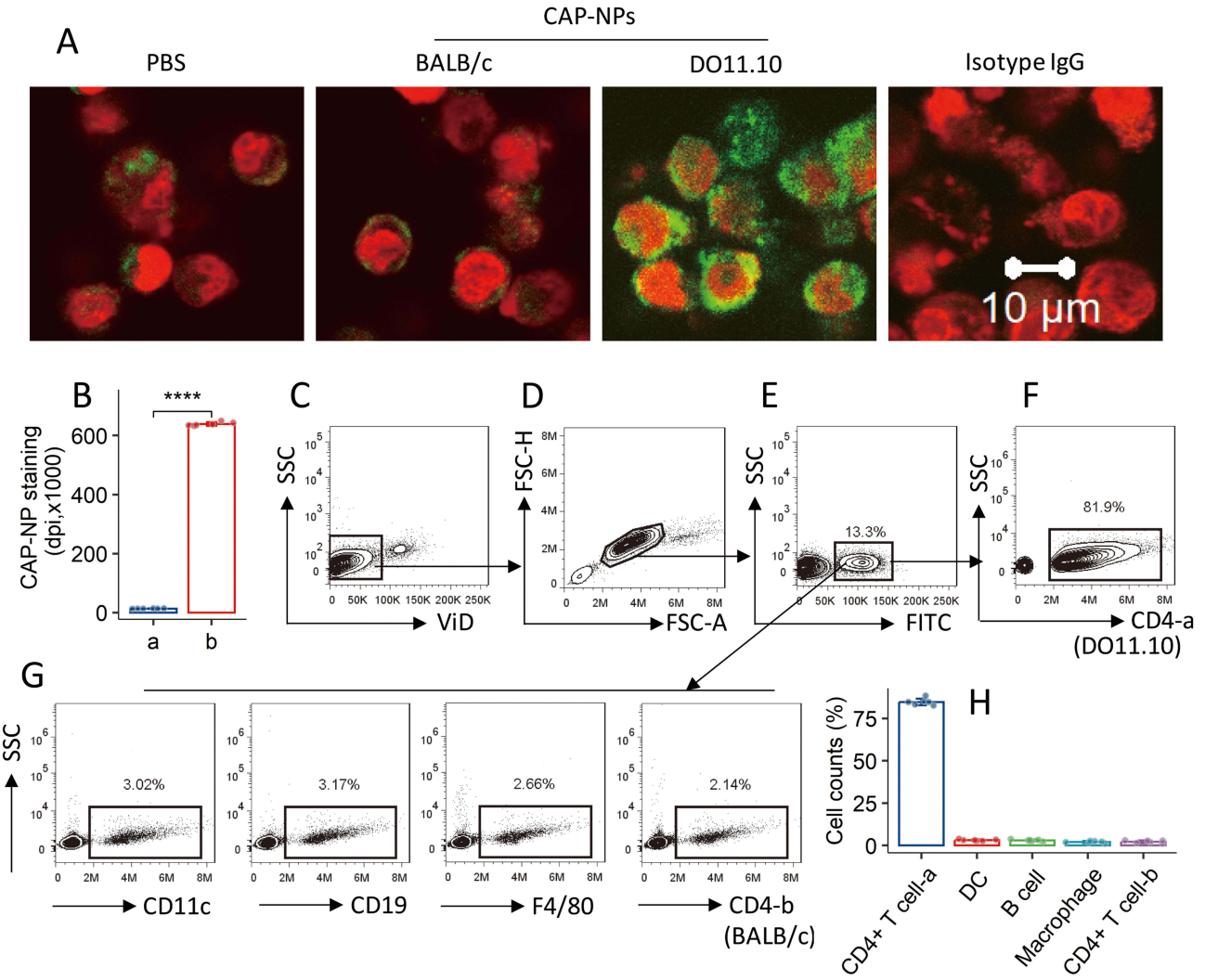

Figure 2 Assessment of CAP-NPs bind to antigen specific CD4$^+$ T cells. (**A** and **B**) CD4$^+$ T cells were isolated from the spleen of DO11.10 mice and BALB/c mice, and exposed to FITC-CAP-NPs (1 μg/mL) in culture for 2 h. Representative images show CAP-NP-binding (in green) on the surface of DO11.10 CD4$^+$ T cells. Bras show the quantity of green color in the images. (**C-H**) mice received nasal instillation (20 μL/nostril; containing FITC-CAP-NPs 5 mg/mL). Single cells of the lungs were prepared and analyzed by flow cytometry. (**C**) dead cells were gated out. (**D**) adherent cells were gated out. (**E**) counts of FITC-positive cells (bound by CAP-NPs). (**F-G**) CAP-NP positive cell type counts. (**H**) bars show CAP-NP bound cell type counts. The data of bars are presented as mean ± SD. Each dot presents one sample. ViD: An active amine fluorescence dye used to stain dead cells. ****p<0.0001 (*t*-test).

Abbreviation: DPI, Dot per inch.

CAP-NPs Induce Airway Antigen Specific CD4$^+$ T Cell Apoptosis

DO11.10 mice or BALB/c mice were treated with CAP-NP-containing nasal instillations daily for five days. After sacrifice, single cells were prepared from the airway tissues, and analyzed by flow cytometry. We found that CAP-NPs induced approximately 10% CD4$^+$ T cell apoptosis in DO11.10 mice, but not in those isolated from naïve BALB/c mice. Treatment of OVA-sensitized BALB/c mice with CAP-NP-containing nasal instillations also caused approximately 10% CD4$^+$ T-cell apoptosis. Treatment of mice with control NPs (containing only one or two of the three components of CAP-NPs) did not induce CD4$^+$ T cell apoptosis (Figure 3A–C). The apoptotic CD4$^+$ T cells were also CD44$^+$ (Figure 3D and E; CD44$^+$ is a marker of antigen-specific CD4$^+$ T cell activation[11]). These results demonstrate that the administration of CAP-NPs via nasal instillations can induce antigen-specific CD4$^+$ T cell apoptosis in the airways.

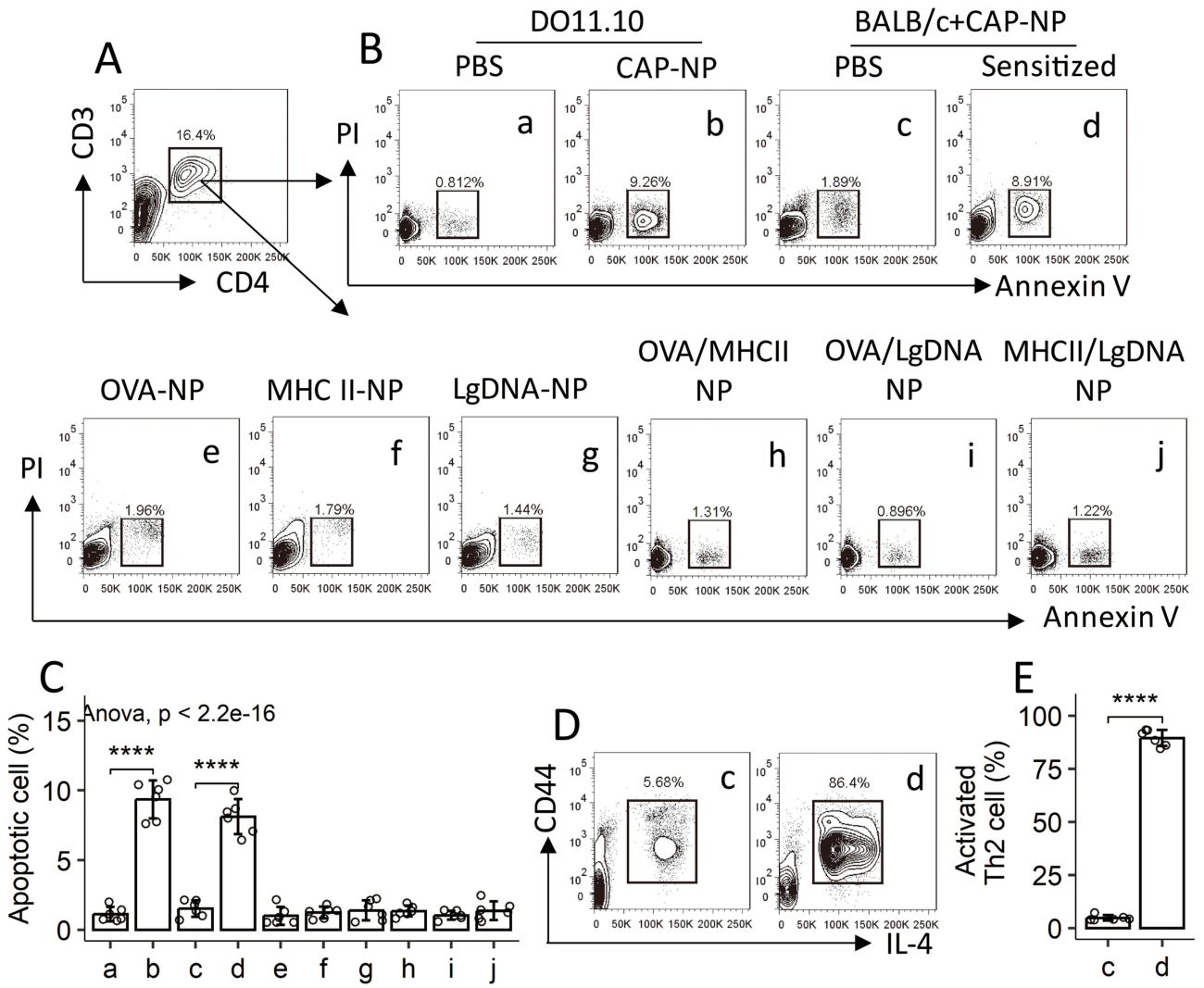

Figure 3 CAP-NPs induce antigen specific Th2 cell apoptosis. DO11.10 mice, naïve BALB/c mice, and OVA-sensitized BALB/c mice received NP-containing nasal instillations daily for 5 days. Single cells were prepared with the lung tissues, and analyzed using flow cytometry. (**A**) $CD4^+$ T cells were gated, from which apoptotic cells were gated (Ba-j). (**C**) bars show apoptotic cell counts from 6 mice per group (The group labels are the same as those in panel (**B**). D, gated plots show that the apoptotic BALB/c $CD4^+$ T cells in panel Bc and Bd are also IL-4^+CD44^+. (**E**) bars show the cell counts of panel (**D**). The data of bars are presented as mean ± SD. Each dot in bars presents one sample. Statistics: ANOVA followed by Bonferroni test (**C**) or Student's *t*-test (**E**). ****$p<0.0001$. NP: Nanoparticle. NPs in panels Be-Bj are control NPs, which only contain the indicated molecules.

CAP-NPs Enhances the Expression of Caspase 8 in Antigen Specific $CD4^+$ T Cells

After exposure to CAP-NPs in culture for 24 h, DO11.10 $CD4^+$ T cells were analyzed by RNA sequencing. Among the differentially expressed genes (DEG) detected, the high expression of *Kdm5a, Casp8, Fas, Fasl*, and lower expression of *Bcl2, Mcl1, Survivin*, and *Mix2* caught our attention (Figure 4A). The data were verified using conventional reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Figure 4B–E). The expression of *Kdm5a* or *Casp8* in $CD4^+$ T cells was positively correlated with apoptotic cells (Figure 4F and G). KDM5A is a demethylase. These results suggest that CAP-NPs induce antigen-specific $CD4^+$ T-cell apoptosis by modulating their epigenetic status.

CAP-NPs Induce Casp8 Gene Promoter Demethylation to Increase Its Expression in $CD4^+$ T Cells

$CD4^+$ T cells were isolated from the DO11.10 mouse spleen and cultured in the presence of CAP-NPs at gradient doses for 48 h. The cells were analyzed by chromatin immunoprecipitation (ChIP). We found an elevated amount of KDM5A in the Casp8 gene promoter locus of CAP-NP-primed $CD4^+$ T cells, in which demethylated H3K9 and demethylated

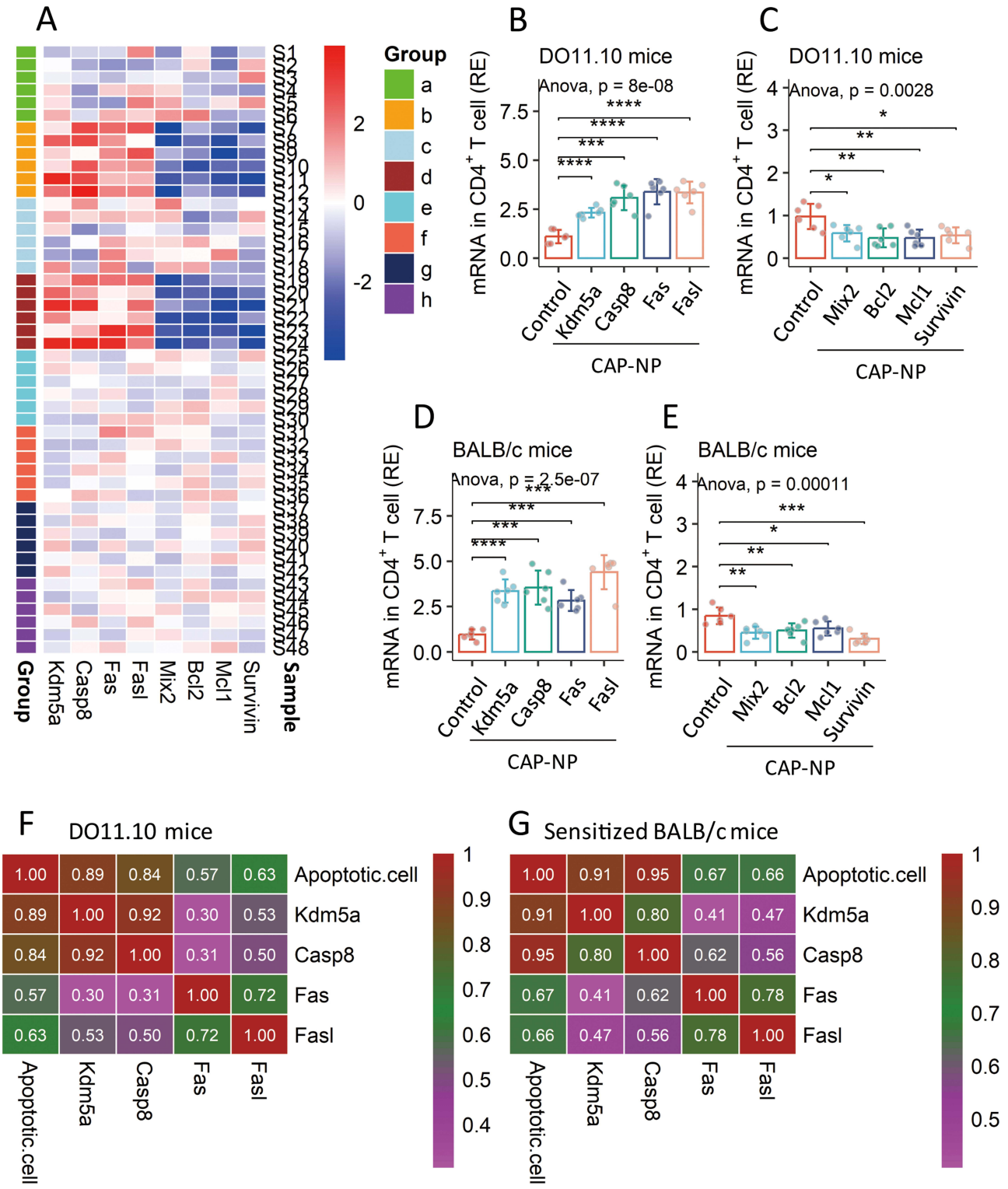

Figure 4 Assessment of CAP-NP-induced apoptosis-associated genes in CD4$^+$ T cells. (**A**) DO11.10 mice and OVA-sensitized BALB/c mice received NP (NP types are the same as those presented in Figure 3)-containing nasal instillations daily for 5 days. CD4$^+$ T cells were prepared with the lung tissues, and analyzed using RNA sequencing. The heatmap shows the levels of DEGs associated with apoptosis. (**B**–**E**), RNA samples were extracted from the CD4$^+$ T cells, and analyzed by RT-qPCR. Bars indicate mean ± SD of mRNA levels of indicated molecules. (**F** and **G**), heatmaps show the correlation coefficients between airway apoptotic cell counts and indicated molecules in CD4$^+$ T cells. Each dot in bars presents one sample. Statistics: ANOVA followed by Dunnett's test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Group labels of panel A are the same as those in panel B of Figure 3. Control: RNA samples are from naïve CD4$^+$ T cells.

Abbreviation: DEG, Differentially expressed gene.

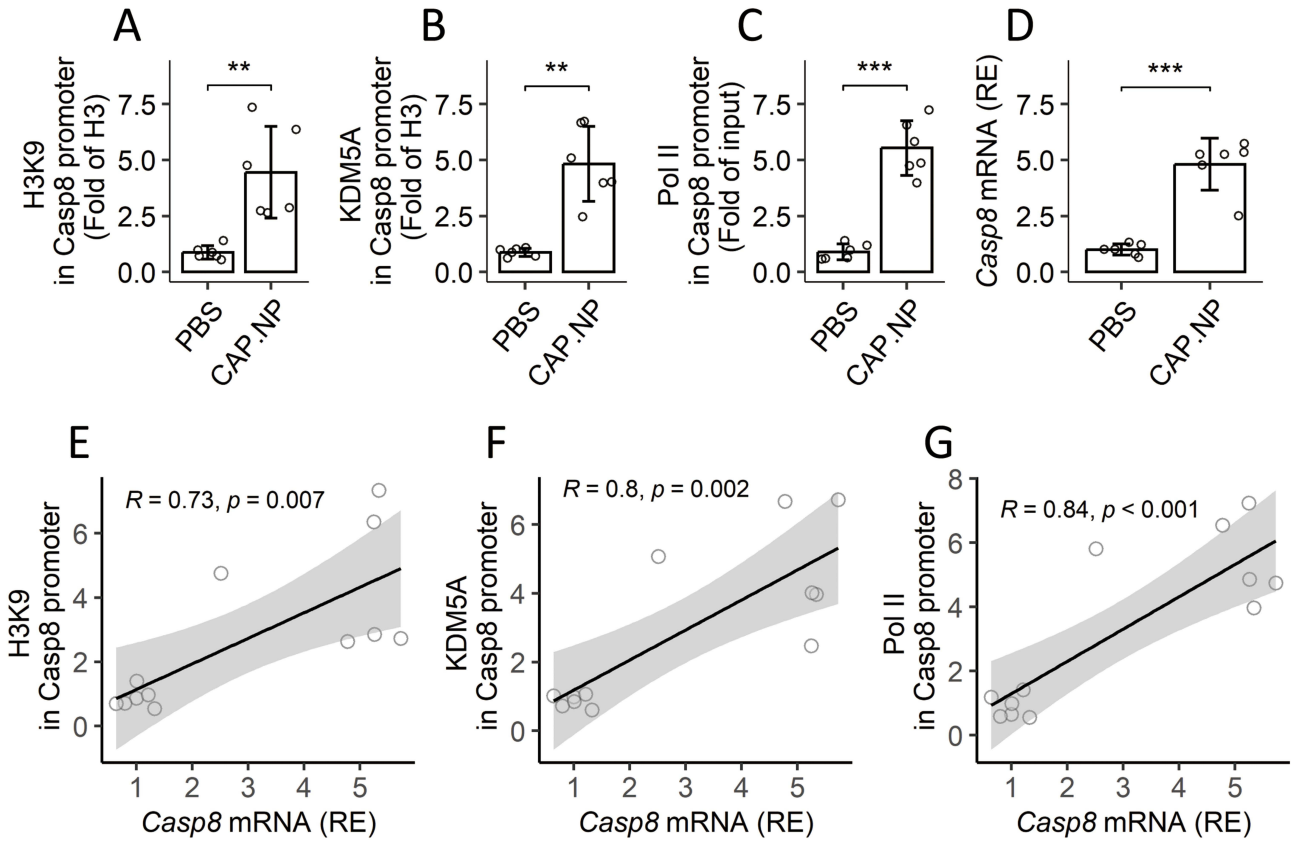

Figure 5 CAP-NPs promote the gene transcription of Casp8 in antigen-specific $CD4^+$ T cells. (**A** and **B**), $CD4^+$ T cells were isolated from the DO11.10 mouse spleen, and exposed to CAP-NPs (1 μg/mL) in culture for 48 h. (**A**–**C**), bars show the indicated molecules in the *Casp8* gene promoter locus. (**D**) the quantity of *Casp8* mRNA in $CD4^+$ T cells. (**E**–**G**) correlation between *Casp8* mRNA and indicated molecules in $CD4^+$ T cells. The data of bars are presented as mean ± SD. Each dot in bars presents one sample. Statistics: Student's *t*-test (**A**–**D**) and Pearson correlation coefficient test (**E** and **F**). $^{**}p<0.01$; $^{***}p<0.001$.

Casp8 promoters were enhanced (Figure 5A and B). Consequently, elevated gene transcription activity of *Casp8* was detected in $CD4^+$ T cells (Figure 5C). The amount of mRNA in $CD4^+$ T cells also increased (Figure 5D), which was negatively correlated with the quantity of H3K9me3, positively correlated with KDM5A, or Pol II in the *Casp8* gene promoter locus (Figure 5E–G). These results demonstrated that CAP-NPs can increase the expression of casp8 in antigen-specific $CD4^+$ T cells by regulating the demethylation of Casp8.

CAP-NPs Reconcile Th2 Polarization in the Mouse Airways

An airway Th2 polarization mouse model was established according to previously published procedures.[12] Elevated levels of Th2 cytokines, including IL-4, IL-5, IL-13, and IL-9, were detected in BALF. After completion of sensitization, the mice were treated with CAP-NP (or PBS)-containing nasal instillations daily for five days. The quantity of Th2 cytokines in BALF was significantly attenuated by CAP-NPs. This effect was abolished by the concurrent administration of IETD-CHO, an inhibitor of CASP8[13] (Figure 6). These results demonstrate that CAP-NPs can attenuate Th2 polarization in the airway.

Discussion

This paper reports the construction of a new nanoparticle (NP), designated CAP-NP. NPs contain a specific antigen, $OVA_{323-339}$, MHC II, and LgDNA, which form NPs through disulfide bonds. Reconciliating Th2 polarization in the airways can be achieved using CAP-NPs.

Drugs in liposomes can be designed to be released slowly or rapidly, depending on their therapeutic purpose. Several drugs or molecules can be incorporated into one liposome to exploit the synergistic effects of drugs. These drugs may not

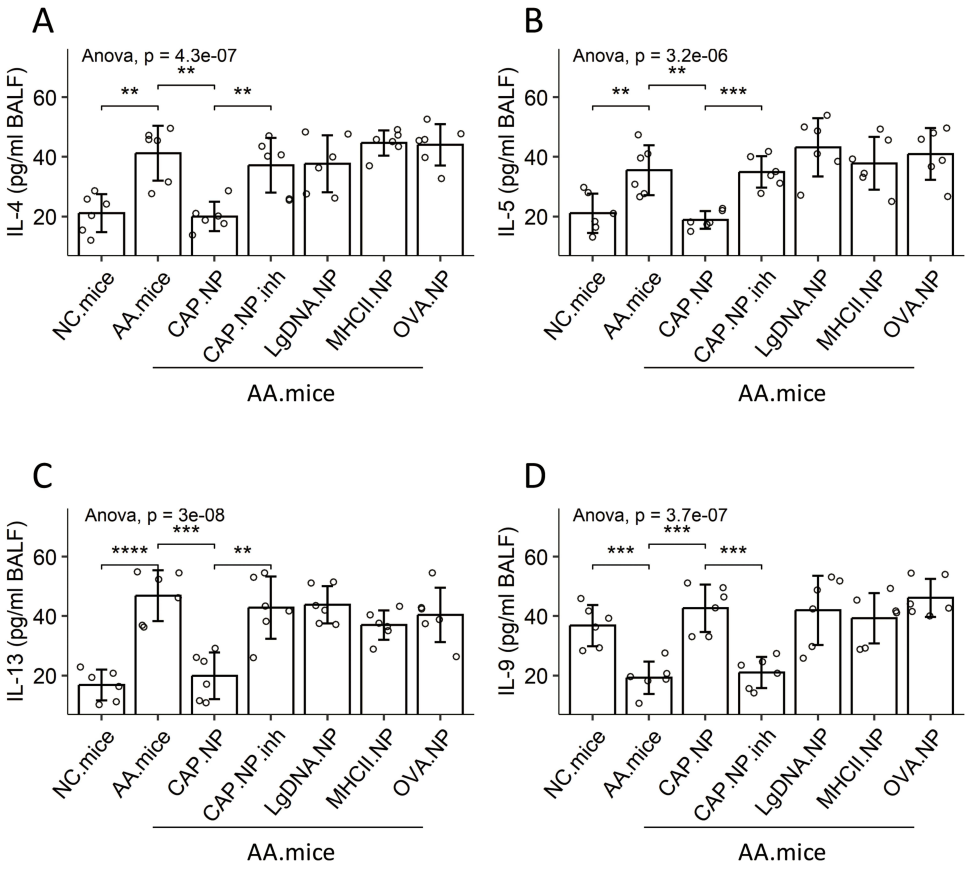

Figure 6 CAP-NPs suppress airway Th2 polarization. An airway allergy (AA) mouse model was established. BALF was collected from mice upon the sacrifice and analyzed using ELISA. Bars show the quantity of IL-4 (**A**), IL-5 (**B**), IL-13 (**C**), and IL-9 (**D**) in BALF. The data of bars are presented as mean ± SD. Each dot in bars presents one sample. Statistics: ANOVA followed by Bonferroni test. ***p*<0.01; ****p*<0.001; *****p*<0.0001. inh: An inhibitor of CASP8 (IETD-CHO; 10 mg/kg, i.p). LgDNA.NP, MHCII.NP, and OVA.NP are control NPs, which only contain the indicated molecules.

be simultaneously released from liposomes because they are only encapsulated in liposomes and not connected to each other.[14] The CAP NPs in the present study could also carry several molecules or drugs. The engineering merits of CAP-NPs are the disulfide bonds that connect molecules. NPs constructed with disulfide bonds are not easily disassembled. Our data showed that the CAP-NPs remained stable for a long time. It did not disassemble in solution at pH7.2 at least for 96 h. This feature allows the conjugated molecules to reach the targeted cells simultaneously.

CAP-NPs are made up of a peptide carrier and three functional substances: a specific antigen molecule OVA, MHC II, and LgDNA. The peptide carrier design was based on data reported by Wang (Cys-Trp-Trp-Arg8-Cys-Arg8-Cys-Arg8-Cys).[1] Our design replaced three amino acids with Pro (Cys-Trp-Pro-Arg8-Pro-Arg8-Pro-Arg8-Cys) to promote its adhesion property.[2] The antigen-MHC II complex (MHC II and OVA) binds specifically to antigen-specific cells. Thus, OVA-specific Th2 cells were the only cell type that selectively bound to CAP NPs in this study. Antigen-specific Th2 cells are major immune cells involved in the development and maintenance of allergic diseases.[15] The decreased or depleted levels of this fraction are expected to mitigate or cure allergic diseases. It seems that CAP-NPs are capable of accomplishing this task. Our data showed that CAP-NPs could specifically induce antigen-specific $CD4^+$ T cell apoptosis.

LgDNA is a component of CAP-NPs. It was extracted from the probiotic strain, *Lactobacillus rhamnosus* GG. This probiotic strain has immunoregulatory functions. For example, it can protect the epithelium of the gastrointestinal tract, inhibit some bacterial strains, promote the Th1 response, and improve immune regulation by facilitating the production of IL-10.[16] The present data show that DNA also has immunoregulatory functions. With the aid of an antigen-specific targeting device, CAP-NPs and LgDNA successfully induced antigen-specific Th2 cell apoptosis in the airways of mice with airway allergies.

$CD4^+$ T cells express Toll-like receptor 9 (TLR9). Activation of TLR9 provides signals secondary to the TCR to modulate $CD4^+$ T cell proliferation.[17] By interacting with CD40, TLR9 promotes the production of anti-parasite cytokines.[17] DNA can bind to TLR9 and modulate targeted cell activities.[18] Our data showed that LgDNA-containing CAP-NPs activated antigen-specific Th2 cells and induce the expression of CASP8. CASP8 is an initiator of apoptosis.[19] Our data are in line with previous reports by showing that CAP-NPs specifically induce apoptosis in OVA-specific $CD4^+$ T cells. Antigen-specific Th2 polarization in local tissues is the pathological basis of airway allergy.[20] Current data suggest that CAP-NPs can be used to reconcile the antigen-specific Th2 polarization in the airways, and have the potential to be used for the treatment of allergic diseases in the airways.

By analyzing CAP-NP-treated antigen-specific $CD4^+$ T cells using RNA sequencing, we found that the expression of *Casp8* and *Kdm5a* was elevated. Both molecules were positively correlated with apoptotic cell counts in the airways of DO11.10 mice treated with CAP-NP and sensitized BALB/c mice. These data suggest that CAP-NPs induce the expression of *Casp8* and *Kdm5a* in $CD4^+$ T cells. KDM5A is a demethylase. It is also involved in the regulation of histone demethylation and gene transcription.[21] We also observed a change in KDM5A and H3K9 levels at the *Casp8* gene promoter locus. These data suggest that CAP-NPs increase the expression of *Kdm5a*, which in turn modulates H3K9 demethylation and *Casp8* promoter demethylation.

The data showed an increase in the expression of Fas and FasL in $CD4^+$ T cells after exposure to CAP NPs. Previous reports have indicated that caspase 8 may promote the activity of the Fas/FasL system (Carmona-Pérez, 2023 #11). Fas/FasL function is also hindered by caspase 8 inhibitors.[22] Whether CAP-NP-induced caspase 8 expression further induces Fas/FasL expression in $CD4^+$ T cells requires further investigation.

We tested the effects of CAP-NPs on the regulation of Th2 polarization in airways. The results showed that the administration of CAP-NPs through nasal instillations effectively mitigated experimental airway Th2 polarization. It is known that the Th2 polarization status is the canonical feature of airway allergy and other allergic diseases.[20] The current data showed that administration of CAP-NPs successfully attenuated airway Th2 polarization. These results suggest that CAP-NPs have translational potential to be developed as a remedy for the treatment of Th2 polarization-associated diseases.

Conclusions

In this paper, we report a new nanoparticle, CAP-NP, which carries a specific antigen, MHC II, and LgDNA. Administration of CAP-NPs suppressed Th2 polarization in the airways by inducing antigen-specific Th2 cell apoptosis.

Data Sharing Statement

All the data are included in this paper.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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