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ARTICLE TYPE

Intracellular delivery of CII TA genes by polycationic liposomes for suppressed immune response of dendritic cells

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Abstract: MHC II transactivator (CII TA) protein is a requisite for the expression of major histocompatibility complex class II (MHC II) proteins which are principal mediators of immune response. Construction of an effective nanocomplex to suppress expression of CII TA proteins can be a potential strategy for inhibiting unwanted immune response. In this work, a nanocomplex was designed by incorporating pCIITA (CII TA genes within a plasmid) into the polycationic liposomes with an optimal mass ratio at 1:2. The monodisperse nanocomplexes possessed spherical shape and uniform size. Results of cells transfection showed that the nanocomplexes displayed an enhanced ability of intracellular delivery than that of naked pCIITA. Expression of CII TA and MHC II proteins were significantly decreased in the transfected cells. Furthermore, the *in vitro* immune response model based on mixed lymphocyte culture experiment confirmed that the nanocomplexes successfully inhibited immune response of dendritic cells (DCs) through intracellular delivery of CIITA genes. All the results suggested this approach has a great potential in suppression of unwanted immune responses, which relates to many immune diseases.

1. Introduction

Immune response plays critical roles in many immune-related diseases, such as rejection of organ graft[1, 2] and self-immune disease. Prolonged treatment of steroids and conventional immunosuppressor to prevent immune response can induce renal or hepatic toxicity and increase the susceptibility to malignancies or infections[3]. Chan (2013) and Chidgey (2008) stated that the emerging cell therapy has encountered the same problem[4, 5] as well. Therefore, there is a strong unmet need for an alternative strategy to suppress immune response.

The biology studies have demonstrated that immune recognition activates a series of complicated immune response[6, 7]. MHC II proteins, mainly expressed on DCs, play an important role in immune recognition[8-10]. That is, MHC II proteins manage immune response partially. But MHC II proteins are encoded by multiple constitutive genes[11-13], such as HLA-DR, HLA-DQ, HLA-DP and so on. Meanwhile, MHC II proteins also can be expressed by their alleles in an inducible ways. So it is hard to silence MHC II genes via gene interference. To date, CII TA protein has been identified as an absolute requisite for expression of MHC II proteins[14, 15], both constitutive and inducible ones (as shown in Fig. 1). Moreover, many efforts have shown that deletion of CII TA genes lead to loss of MHC II expression and immune-deficiency phenotype[15, 16]. Therefore, CII TA is an ideal gene target for silence of MHC II genes and suppressed immune response of DCs.

RNA interference (RNAi) is a powerful method for reducing unwanted expression of endogenously expressed proteins[17], such as CII TA protein here. Now, short hairpin RNA (shRNA) was often used as the siRNA[18]. Though the shRNA facilitated itself take effect in a longer time[19], degradation of genes in the bio-environment can't be avoided. Recently, the combination of genes with cationic polymer has been widely investigated[20-23] to enhance stability of genes in the biological solutions. The cationic polymers were commonly used amino-enriched molecules, such as polylysine, chitoson, PEI and their derivatives. Among various polymercationic vectors, liposomes draw much attention due to high delivery efficiency[24, 25].

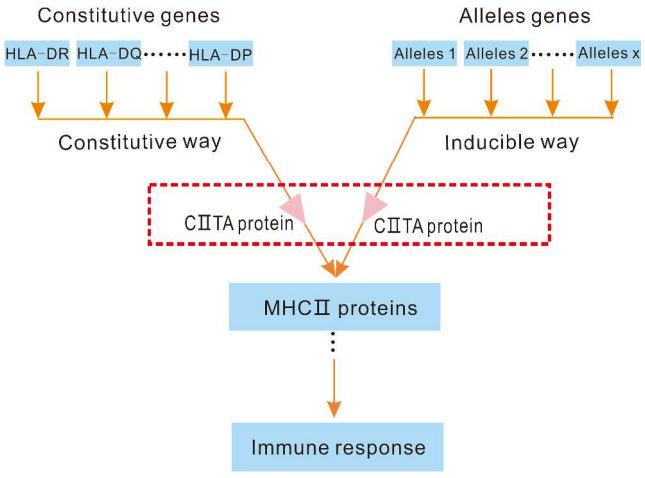

Fig.1 CII TA protein considered as controller of MHC II expression. MHC II proteins are expressed not only by their constitutive genes but also by their alleles in an inducible way. CII TA protein controls both expression of MHC II in two ways because of promotion effect for MHC II genes. The as-synthesized MHC II proteins were involved in immune responses.

In our previous work[26, 27], we have successfully synthesized polycationic liposomes based on octadecyl-quaternized lysine modified Chitosan (OQLCS). Negatively charged genes interacted with the polycationic liposomes via electrostatic interactions to form nanocomplexes. The nanocomplexes possessed a number of advantages: 1) Because of electrostatic repulsion against aggragation the nanocomplexes were in a stable monodisperse state. 2) Optimal nanocomplexes could be obtained by varying the mass ratio between genes and liposomes. 3) Amino groups on the liposomes can condense genes and contributed to high transfection efficiency.

We developed pCIITA/liposomes nanocomplexes, based on polycationic liposomes (OQLCS), as vectors for genes intracellular delivery. We draw a schematic illustration for the working principle of nanocomplexes to suppress immune response of DCs, as shown in Fig.2. After pCIITA was delivered into cells, pCIITA would produce siRNA that targeted mRNA of CIITA for degradation. Thus, synthesis of CII TA proteins was suppressed. And then MHC II proteins, whose synthesis needed CII TA proteins as promoters, were inhibited as well. Finally, the immune recognition involved MHC II proteins was blocked. EGFP, here, was used as surrogate marker for exogenously administered pCIITA.

In this work, the shRNA of CII TA genes was synthesized based on design principles of siRNA and was inserted into a plasmid as a segment. The plasmids were called pCIITA for short. Agarose gel electrophoresis (AGE) was carried out to screen the optimal mass ratio between pCIITA and polycationic liposomes. Expression of CII TA and MHC II both on genes and protein levels was estimated though *in vitro* cells transfection. Furthermore, one way mixed lymphocyte culture (MLC) experiment, an *in vitro* immune response model, was performed to confirm the suppressed immune response of DCs transfected by nanocomplexes.

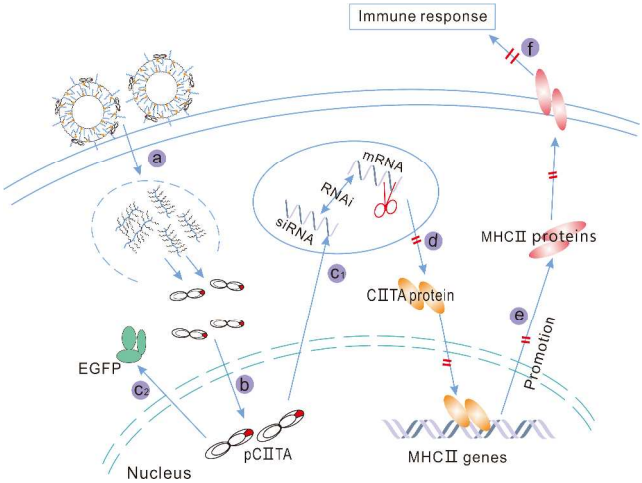

Fig.2 The schematic illustration for the designed nanocomplexes to suppress immune response. a: The pCIITA were delivered into cells by nanocomplexes. b: The pCIITA escaped from endosomes and came to nucleus. c1: The shRNA in pCIITA produced siRNA to target mRNA of CII TA for degradation. d: Without mRNA, synthesis of CII TA protein was blocked. e: Because of absence of CII TA protein, promotion for MHC II genes was blocked, resulting in suppressed expression. f: Immune response that involved with MHC II proteins was inhibited. c2: EGFP was considered as surrogate marker for intracellular delivery of pCIITA.

2. Materials and methods

2.1 Materials

The pGenesil-1, restriction enzyme and antibodies used in the experiment were purchased from Wuhan Genesil Bio-technology Co., Ltd (China). Cationic polymer OQLCS was prepared in our laboratory[26, 27]. Cholesterol was purchased from Tianjin chemical reagent factory (China). Lipotamine 2000 was purchased from Invitrogen (USA). Wistar rat DCs and SD rat lymphocytes were obtained from Experimental Animal Center of the Radiomedicine Reseach Institute, Chinise Academy of Medical Sciences. IL-2 ELISA test kit and IFN-γ ELISA test kit were purchased from Shangbo biomedical technology Co., Ltd (China).

2.2 Construction of pCIITA

A recombinant plasmid was constructed by inserting shRNA of CII TA into the Bam HI and Hind III restriction sites of I pGenesil-1 plasmid, called pCIITA for short. The shRNA of CII TA used to interfere with CII TA genes was synthesized in a specific sequence: 5′-AGGCTGAGCTTGTACAACA-3′. In addition, negative control plasmid (pHK) was also constructed by cloning a non-specific sequence (5′-GACTTCATAAGGCGCATGC-3′) into the same restriction site. Restriction enzyme was used to cleave the plasmid, followed by running AGE to confirm the construction of the plasmid.

2.3 Preparation of polycationic liposomes

Briefly, OQLCS and cholesterol (w/w, 2:1) were dissolved in 4 mL chloroform. After chloroform was evaporated using a vacuum rotary evaporator (Shanghai Shensheng Bio-Technology Co. Ltd., China), a thin film of the polymer was formed. The lipid film was dispersed in 10 mL distilled water and sonicated for 10 min. Subsequently, 0.5 mg SPDP was added to the lipid solution. After dialyzed for purification, 1 mL TAT solution (0.01 M) was added. The mixture was incubated at 4 ℃ overnight. The morphology of the cationic polymer liposome was observed by TEM. The average size and zeta potential was determined using a Brookhaven Zetasizer (Brookhaven Instruments Ltd., U.S.).

2.4 Screening for the optimal pCIITA/liposomes nanocomplexes

The pCIITA (1 μg) were incubated with liposomes at different mass ratio of 1:0, 1:0.5, 1:1, 1:2, 1:2.5, 1:3, respectively. The optimal nanocomplexes were screened out by AGE and were used in the all subsequent experiments.

2.5 Estimate for delivery efficiency of nanocomplexes *in vitro* cells transfection

After Rats' myeloid DCs were cultured for 24 h, cells resuspened with complete medium (Dulbecco's minimal essential medium with 10% fetal calf serum, 1% penicillin and streptomycin) at a concentration of 1×10^5 cells/mL were seeded on the plates. 2 μg naked pCIITA, 1 μL Lipofectamine 2000 and 2 μg nanocomplexes in 50 μL Opti-MEM were added into wells for cells transfection, respectively. 50 μL Opti-MEM were used for control. After incubating for 48h, the transfection efficiency was estimated by fluorescence microscope and flow cytometry (FAC-Scalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.6 Expression of CII TA and MHC II in transfected cells

1 mL Trizol and 0.2 mL $CHCl_3$ was added to the transfected cells as lysis buffer. The mixture was centrifuged three times with alcohol to get mRNA extract. Then the mRNA extract was used for reverse transcription to synthesized cDNA of CII TA and MHC II, respectively. Then the cDNA was amplified and analyzed with real time-PCR (Bio-Rad, Hercules, CA).

After phycoerythrin (PE)-conjugated anti-rat MHC II antibody was used to incubate with DCs for 30 min, the intensity of PE on the cells were analyzed by flow cytometry.

2.7 Investigations on immune response of DCs based on one way MLC

Transfected cells and negative control cells (SD rat lymphocytes) were exposed to mitomycin C solution (1mg/mL) at 37℃ for 30min, followed by centrifugation to stop the reaction. The collected cells were resuspended at a density of 1×10^6 cells/mL respectively, as the stimulant cells. The stimulant cells were mixed with untreated SD rat lymphocytes that act as reactive cells at a ratio of 1:1. After the mixed cells were cultured for 3 days, cell proliferation of SD rat lymphocytes, cytokines (IFN-γ and IL-2) contents in the culture supernatant and expression of CD^{4+} protein were tested for the evaluation of the immune response. MTT method was performed for analysis of cell proliferation as follows: After cells were cultured for 2 days, 20 μL of MTT reagent solution (5g/L) was added into wells and incubated for 4 h with cells. Discard medium in the wells, 200 μL of DMSO was added into each well. Upon shaken for 10 min, absorbance at 570 nm was record. Culture supernatant was collected for the cytokines (IFN-γ and IL-2) analysis, using enzyme linked immunosorbent assay (ELISA) with IFN-γ and IL-2 ELISA Kit (Bender MedSystems, Burlingame, CA, USA), respectively. The operation procedure was carried out according to the specification of ELISA. To determinate the CD^{4+} expression, Phycoerythrin (PE)-conjugated anti-rat CD^{4+} antibody was used to mark CD^{4+} proteins on cell surface. After the residual antibody was washed, the cells were detected by flow cytometry analysis.

3. Results and discussions

3.1 Characterization of the pCIITA/liposomes complex

The CII TA complementary shRNA was inserted into the plasmid (pGenesil-1) to construction a relative stable form (as shown in Fig. 3a). The as-synthesized plasmid was named as pCIITA. The linkage of the shRNA to the plasmid was corroborated by restriction enzyme digestion. The Sac I sites both in pGenesil-1 and shRNA would be cut by enzyme, resulting in a segment with 900 bp. Compared with DNA marker, DL 2000, we could see a band at ~900 bp in pCIITA (Fig. 3b), which confirmed the successful formation of pCIITA.

To enhance the intracellular delivery efficiency of naked plasmid, the combination of genes and polycationic liposomes has been widely investigated. The liposomes (OQLCS) we used in this study were successfully synthesized in our previous work as shown in Fig. 4a and 4b. As depicted in Fig.4c, OQLCS and cholesterol were assembled to be nanostructures though hydrophobic interactions. Then positively charged nanostructured liposomes condensed the negatively charged pCIITA to form pCIITA/liposomes nanocomplexes via electrostatic interaction. Due to influences of charge density, properties of the

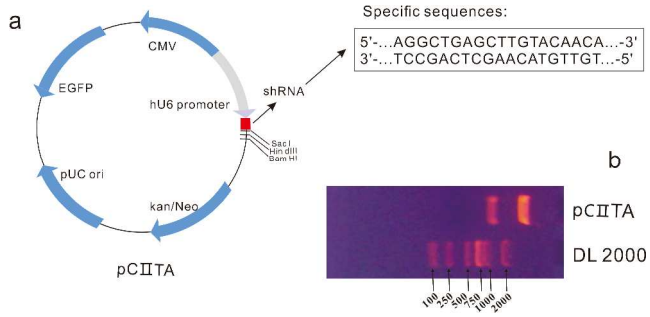

Fig. 3 a) Schematic design of pCIITA. The shRNA containing specific sequences of CII TA was inserted into the plasmid. b) The image of restriction enzyme digestion: DL 2000 was a DNA marker.

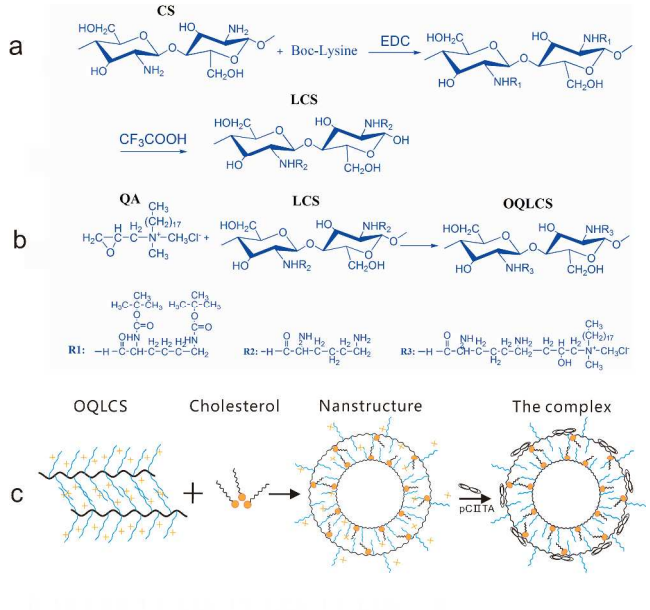

Fig.4 a) Modification of chitosan. b) The synthesis of OQLCS. c) The schematic representation of pCIITA/liposomes formation. Amphiphilic OQLCS assembled to be nanostructure with cholesterol used as a stabilizer. The pCIITA interacted with the liposomes nanostructure through electrostatic interactions to form the nanocomplexes.

nanocomplexes would be different with various mass ratio between the two components. AGE was performed to screen the optimal nanocomplexes. As shown in Fig. 5a, when the mass ratio came to 1:2 or higher than 1:2, no separated plasmid was observed any more, which indicated a completed cage of plasmids. This mass ratio of 1:2 was selected as the optimal and used in all the subsequence experiments.

The morphology of the nanocomplexes was observed by TEM. Fig. 5b clearly showed that the monodisperse nanocomplexes possessed a uniform size less than 100 nm. Dynamic light scattering (DLS) was used to characterize average size and zeta potential of the liposomes and the nanocomplexes (Fig. 5c and 5d). The average diameter of the nanocomplexes was a little smaller than that observed by TEM. This may be caused by different mechanism of two methods. Samples measured by DLS were in solution state, resulting in larger size because of hydrated ionic radius. The diameter of the liposomes changed a lot before combined with pCIITA and after. As the surface zeta potential of liposomes changed a lot, from 50 mV down to 30 mV, we deduced that there was a strong electrostatic interaction between

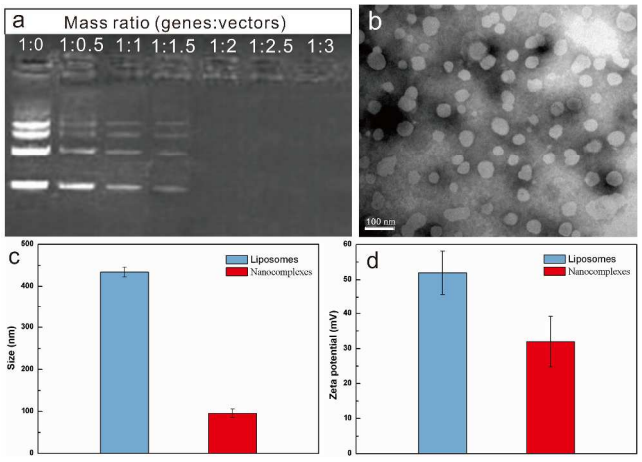

Fig.5 a) The nanocomplexes with different mass ratio of pCIITA to liposomes were optimized by AGE. b) TEM image of the optimal nanocomplexes. c) Average size of the liposomes and optimal nanocomplexes. d) Zeta potential of the liposomes and the optimal nanocomplexes.

polycationic liposomes and pCIITA when they form nanocomplexes. Just because of this interaction, the stretched polycationic liposomes were condensed to be tightly by the negatively charged pCIITA, resulting in nanocomplexes with smaller diameter than pure liposomes. The change of size and zeta potential confirmed the formation of the nanocomplexes.

3.2 Intracellular delivery of the nanocomplexes into DCs

We investigated the intracellular delivery efficiency of the pCIITA/liposomes nanocomplexes into DCs. The expression of EGFP was used as a surrogate marker for intracellular delivery of pCIITA. Transfected cells were observed by fluorescence microscope. Only when pCIITA was delivered into cells can EGFP genes express their proteins. From Fig.6, we can see obvious green light emitted by EGFP in the transfected cells, which indicated that pCIITA was successfully delivered into cells. After preliminary confirmation, flow cytometry was used for quantitative analysis. Compared to the naked pCIITA, the nanocomplexes tripled in intracellular delivery efficiency, as showed in Fig. 7a. Lipofectamine was a commercial reagent that was often used for transfection. Results showed no distinct difference between the cells transfected with Lipofectamine and with nanocomplexes. The transfection results showed that nanocomplexes achieved high intracellular delivery efficiency.

3.3 Suppressed expression of CII TA and MHC II in DCs

In order to estimate the suppression effect of the nanocomplexes, expression of CII TA and MHC II both on gene and protein level were tested. Pure liposomes and non-specific pHK/liposomes nanocomplexes were set as controls. Real time-PCR was carried out to analyze transcription of the two genes. As shown in Fig. S4, synthesis of CII TA and MHC II mRNA decreased greatly in the cells transfected with pCIITA/liposomes nanocomplexes when compared to the controls. This result revealed that not only transcription of CII TA but also that of MHC II was inhibited. MHC II proteins on the cell surface were labeled by PE-conjugated anti-rat MHC II antibody.

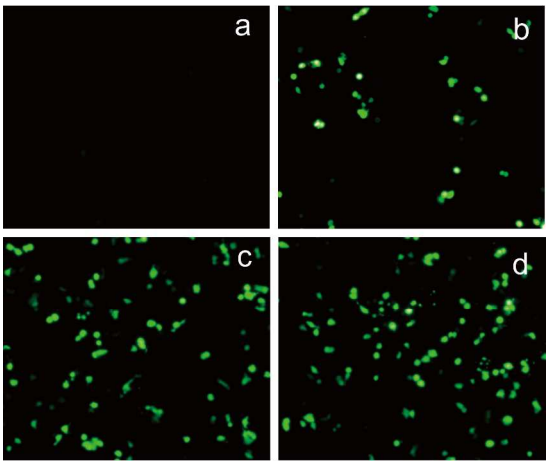

Fig.6 Fluorescence microscopy image of DCs transfected with a) blank media, b) naked pCIITA, c) lipofectamine, and d) pCIITA/liposomes.

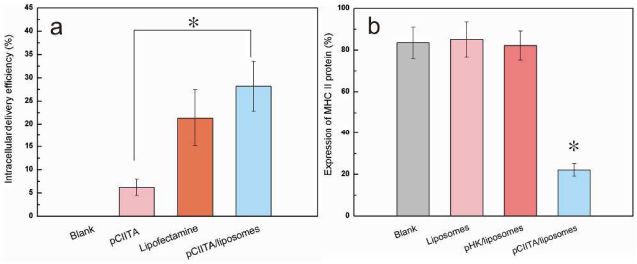

Fig.7 a) Intracellular delivery efficiency of the pCIITA measured in the transfected DCs. The data are the mean ± SE (n = 6). *P < 0.01 as compared to the cells treated with naked pCIITA (ANOVA). b) Expression level of MHC II proteins in transfected DCs. The data are the mean ± SE (n = 6). *Significantly lower than other groups (P < 0.01, ANOVA).

Flow cytometry read the fluorescence intensity of PE and gave the consisting results of decreased expression of MHC II proteins shown in Fig. 7b. The pCIITA/liposomes nanocomplexes significantly down-regulate the expression of MHC II protein, while the controls had no effect. These results suggested that the pCIITA/liposomes nanocomplexes efficiently suppressed the expression of MHC II and caused deficiency-MHC II DCs.

3.4 Investigations on suppressed immune response of DCs

MLC is a common *in vitro* model of immune response, cell proliferation, cytokines secretion and phenotype change was three indicators for the estimate. In MLC experiment, Wistar rat DCs was recognized by SD rat lymphocytes via MHC II molecules. This immune recognition would stimulate lymphocytes to proliferation, secretion of cytokines and phenotype change. We expected that the deficiency-MHC II Wistar rat DCs would exhibit low immunogenicity when mixed with SD rat lymphocytes. In order to confirm the hypothesis, we took three indicators tested to estimate for the immune response.

Lymphocytes proliferation assay presented in Fig. 8a showed that pCIITA/liposomes nanocomplexes group had a low value of 0.4125±0.0561, which was similar to the negative control, whereas the positive control had a value more than 0.8. This indicated that deficiency-MHC II DCs can effectively reduce the stimulating effect on proliferation of lymphocytes. Then the test of cytokines IFN-γ and IL-2 secreted by lymphocytes were performed by ELISA. Fig. 8b and Fig. 8c showed the content of IFN-γ and IL-2 in culture supernate, respectively. Compared to

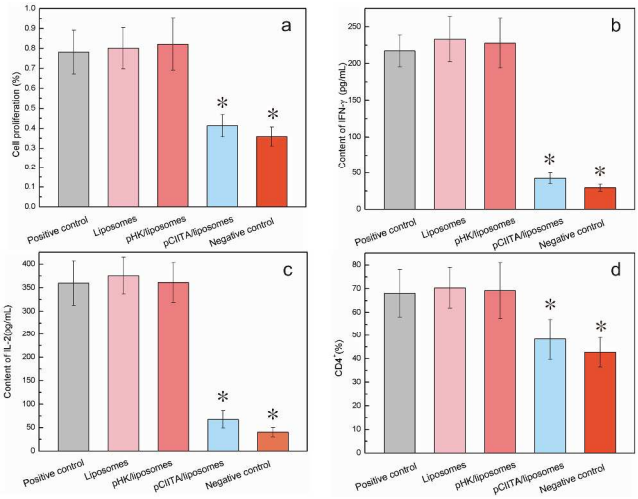

Fig.8 The level of immune response was estimated. a) Cell proliferation of stimulated lymphocytes. b) and c) Cytokines secreted by stimulated lymphocytes. d) Expression level of CD4+ on surface of stimulated lymphocytes. The data are the mean ± SE (n = 6). *P < 0.01 as compared with cells in positive control, liposomes and pHK/liposomes groups (ANOVA).

the positive control, there was a clear decrease of both IFN-γ and IL-2 in the pCIITA/liposomes nanocomplexes group. Because of interaction with MHC II proteins, the phenotype of SD rat lymphocytes would be changed. It has been reported that MHC II protein can induce increase of CD^{4+} on lymphocytes surface[3]. As we expected, the results showed an obvious reduction for expression of CD^{4+} in the pCIITA/liposomes group. Taken together, all the results in one MLC confirmed that pCIITA/liposomes nanocomplexes inhibited immune response and contributed to the low immunogenicity of DCs.

4. Conclusions

In this study, we designed pCIITA/liposomes nanocomplexes for intracellular delivery of pCIITA to suppress immune response. Charge density played a critical role in the formation of pCIITA/liposomes nanocomplexes. AGE experiment was displayed to get the optimal mass ratio of 1:2 between pCIITA and liposomes. *In vitro* cells transfection revealed that the polycationic liposomes enhanced intracellular delivery efficiency of naked pCIITA. Because of RNA interference, pCIITA delivered into the cells significantly suppressed the expression of CII TA and MHC II genes in DCs. Thus, immune response that needed MHC II proteins to complete immune recognition was blocked. As we expected, the results of lymphocytes proliferation, cytokine secretion and phenotype change showed a lower level for immune response in pCIITA/liposomes nanocomplexes group. These results demonstrated the potential of pCIITA/liposomes nanocomplexes as a high efficiency approach for suppressed immune response.

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