RESEARCH ARTICLE

Evaluation of two different adjuvants with immunogenic uroplakin 3A-derived peptide for their ability to evoke an immune response in mice

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ABSTRACT. ***Rationale:*** **Organ- or tissue-specific antigens produced by normal tissue or by cancer cells could be used in cancer immunotherapy, to target the tumor. In our previous study, we induced T-cell-mediated, bladder-specific autoimmunity by targeting the bladder-specific protein Uroplakin 3A (UPK3A). UPK3A is a well-chosen target for developing an autoimmune response against bladder cancer since the antigen is also expressed in bladder tumors. To use this peptide, which was derived from the UPK3A protein in a bladder cancer vaccine study, it is necessary to induce a strong immune response. In this study, we aimed to develop a robust immune response in BALB/c mice using the well-characterized keyhole limpet hemocyanin (KLH)-conjugated peptide antigen (UPK3A 65-84) conjugated with an immunogenic carrier protein. In combination with the peptide, we used either Freund's complete adjuvant (CFA) or CpG (cytosine-phosphate-guanine oligonucleotides) as effective adjuvants in order to overcome tumor tolerance.** ***Objectives:*** **The immune response evoked by UPK3A 65-84 peptide, using two different adjuvants, was compared by detection of changes in the proliferative response of immune cells, in the cytokine profile, and in the immune cell populations.** ***Findings:*** **We demonstrated that CpG, combined with KLH-UPK3A 65-84, promoted a more robust immune response, via induction of higher IL-2, IFN-γ, TNF-α, IL-17 production and activation of more immune cells ($CD4^+$ T cells, $CD8^+$ T cells, NK cells CD11b, CD45), than CFA and the KLH-UPK3A 65-84.*** ***Conclusion:*** **CpG as an adjuvant combined with KLH-UPK3A 65-84 could be used in preclinical models of bladder cancer for the development of cancer immunotherapy strategies.**

Keywords: uroplakin 3A, Freund's complete adjuvant, cytosine-phosphate-guanine, Th1/Th2 immune response

Bladder cancer is the fourth most common type of cancer in men, and the ninth most common in women [1]. More than 70% of bladder tumors are non-muscle-invasive, superficial bladder cancers, where transurethral resection (TUR) is used as a standard treatment followed by adjuvant intravesical *Bacillus Calmette-Guerin* (BCG) immunotherapy [2]. Due to the high rate of recurrence and the risk of progression to invasive cancer, there is an urgency to develop more effective treatments for this disease [2, 3].

The use of the immune system to eliminate cancer offers a unique and potentially more powerful approach compared to conventional therapies with cytotoxic agents. Organ- or tissue-specific antigens produced by normal tissue or by cancer cells could be used in cancer immunotherapy to target the tumor [4-7]. Several promising pre-clinical studies have been carried out using highly immunogenic antigens that are tissue-specific and also specifically expressed in cancer types.

Uroplakins are integral membrane proteins of uroepithelium, and are produced specifically in the bladder [8-10]. Uroplakin 3A (UPK3A) has been shown to be highly expressed in bladder cancer in many studies. UPK3A expression is limited to the apical membrane of the superficial umbrella cells of normal urothelium, and its expression is also shown to be persistent in more than 80% of non-invasive, transitional cell carcinoma, in 55% of invasive, transitional cell carcinoma, and in 65% of metastatic, transitional cell carcinoma of the bladder [11, 12]. Recently, we induced both a T-cell response and an antibody response by targeting the bladder-specific, UPK3A protein [13].

In the present study, we selected Freund's complete adjuvant (CFA) and cytosine-phosphate-guanine (CpG) (Toll-like receptor 9 ligand) as adjuvants that are known to trigger Th1-type immune responses and which have been previously used in cancer immune therapy in preclinical models [14-18]. Although a variety of adjuvants are used

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in preclinical models, CFA is the most commonly used for generating immune responses against different antigens. It is a water-in-oil adjuvant composed of mineral oil and inactivated *Mycobacterium tuberculosis* particles to provide the immune stimulation. This adjuvant is used routinely for the production of antibodies against desired antigens, and for the stimulation of Th1-type immune responses [19]. The $CD4^+$ and CD8+ T lymphocyte responses and antibody production have been well-established in different cancer models in mice upon immunization with combinations of the desired antigen and CFA [5, 7, 20].

CpG oligonucleotides containing unmethylated CG nucleotides stimulate the immune system through the TLR9 pathway and generally induce an effective Th1-type immune response [21, 22]. CpG oligonucleotides are used in cancer immunotherapy for inducing CD4+ and CD8+ responses, and for generating antibodies against the tumor-specific antigen to inhibit tumor growth [23-27].

In the present study, we aimed to develop a robust immune response with this well-characterized antigenic peptide in BALB/c mice, in combination with either CFA or CpG as adjuvants to overcome tumor tolerance. Immunization was optimized through the conjugation of UPK3A-derived peptide with the highly immunogenic keyhole limpet hemocyanin (KLH) carrier protein in order to achieve maximum activity. The immune responses evoked by the antigen using these two adjuvants were analyzed thoroughly, and the efficiency of the different adjuvants was evaluated by comparison of proliferative responses, cytokine profile and immune cell content.

METHODS AND MATERIALS

Peptide synthesis and conjugation

We have previously shown that the peptide consisting of the 65-84 amino acid residues of uroplakin 3A protein was highly immunogenic in BALB/c mice [13]. The 20-mer UPK3A 65-84 peptide (AMVDSAMSRNVSVQDSAGVP), which contains the -SXXVXV- binding motif for IAd MHC class II molecules, was synthesized and purified by Thermo Fisher Scientific. The peptides were purified by reverse-phase HPLC, and the amino acid compositions were confirmed by mass spectrometry. The KLH-conjugated UPK3A 65-84 peptide was also synthesized by Thermo Fisher Scientific.

Mice and immunization

All protocols were pre-approved by the Institutional Animal Care and Use Committee of Erciyes University Experimental Research and Application Center (DEKAM) (Licence no: 10/07/2013-07-13/99) in compliance with the Public Health Service policy on humane care and use of laboratory animals.

Female BALB/c mice were purchased from Erciyes University, Experimental Research and Application Center (DEKAM). CFA (Sigma) and ODN 1826 (Invivogen; Version # 11D29-MT), which is a synthetic oligodeoxynucleotide containing unmethylated CpG motifs (*CpG ODNs*), were used as the adjuvants. Mice aged 6-8 weeks were injected subcutaneously (s.c.) in the abdominal flank, with or without 200 μg of KLH-conjugated UPK3A 65-84 peptide in 200 μl of an emulsion of equal volumes of water and CFA containing 400 μg of *Mycobacterium tuberculosis* (Sigma; H37RA, ATCC 25177). Another group of mice were injected with or without 200 μg KLH-conjugated UPK3A 65-84 peptide in 200 μL of an emulsion of equal volumes of water and 2 mg/mL CpG. ODN 1826 VacciGrade^{TM} was dissolved in the endotoxin-free physiological water, aliquoted and then stored at -20°C until used. ODN 1826 VacciGrade^{TM} is sterile and contains no endotoxin (measured by kinetic chromogenic LAL assay).

Proliferation assay

To compare the lymph node cell (LNCs) proliferative response of mice immunized with KLH-conjugated UPK3A 65-84 using different adjuvants (CFA *versus* CpG), the inguinal and axillary LNCs were removed from mice 10 days after immunization (10-day-primed LNC), and cultured for BrdU incorporation assays. The cells were plated at 3×10^5 cells/well in a single-cell suspension in 96-well, flat-bottomed microtiter plates (Falcon, BD Biosciences) with Dulbecco's modified Eagle medium (DMEM) (Lonza) containing 10% fetal bovine serum (Biochrom), 5% HEPES buffer (Lonza), 2% L-glutamine, and 1% penicillin/streptomycin (Biochrom). Serial, 10-fold dilutions of peptide or 2 μg/mL of anti-mouse CD3 (BD Biosciences; positive control) were added to triplicate wells and the cells were incubated at 37°C for 72 hours in humidified air with 5% CO_2. Cell proliferation ELISA, BrdU (colorimetric) (Roche), was used to assess the proliferation of UPK3A 65-84-stimulated cells. The assay protocol was conducted according to manufacturer's instructions. Briefly, the cells grown in 96-well, tissue-culture microplates were labeled by the addition of 20 μL/well BrdU (10 μM) that is incorporated in place of thymidine into the genomic DNA of proliferating cells over 24 hours. After removing the labeling medium, the cells were fixed, and the DNA denatured in one step by adding 200 μL/well FixDenat. After removing FixDenat, the anti-BrdU-POD antibody was added. The immune complexes were detected by the substrate reaction. Finally, the color development was quantified by measuring the absorbance at 450 nm using an ELISA plate reader (Readwell Touch-Robonic).

Cell culture

Five weeks after immunization of female BALB/c mice with 200 μg KLH-conjugated UPK3A 65-84 with CFA, and 200 μg KLH-conjugated UPK3A 65-84 with CpG, splenocytes were teased into single-cell suspensions. The cells were seeded at a density of 5×10^6 cells per well in a 24-well, flat-bottomed plates (BD Biosciences) in a total volume of 2 mL/well in DMEM (Lonza) supplemented with 10% fetal bovine serum (Biochrom), 5% HEPES buffer (Lonza), 2% L-glutamine, and 1% penicillin/streptomycin (Biochrom). The splenocytes were activated by the addition of 50 μg/mL peptide *in vitro* for 72 h at 37°C in humidified air with 5% CO_2. After 72h incubation, the cells were placed in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA isolation.

In another set of experiment, five weeks after immunization, the cells isolated from the spleen were cultured in 24-well, flat-bottomed falcon plates in the pres-

Table 1
Primer lists

Gene	Primer F	Primer R
Beta-actin	GGTCATCACTATTGGCAACG	ACGGATGTCAACGTCACACT
Interferon-γ	TGATGGCCTGATTGTCTTTCA	GGATATCTGGAGGAACTGGCAA
TNF-alpha	CGAGTGACAAGCCTGTAGCC	GTGGGTGAGGAGCACGTAGT
IL-2	GCAGGCCACAGAATTGAAAG	TCCACCACAGTTGCTGACTC
IL-4	CCTCACAGCAACGAAGAACA	ATCGAAAAGCCCGAAAGAGT
IL-10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
NKRP1A	GGCTTGGCATGAGTCACC	TTCAGAGCCAACCTGTGTGA
CD11b	TGTGAGCAGCACTGAGATCC	CAGCAGTGATGAGAGCCAAG
CD11c	ATTTCTGAGAGCCCAGACGA	CCATTTGCTTCCTCCAACAT
CD45	GGGTTGTTCTGTGCCTTGTT	CTTGCCTCCATCCACTTCAT

ence of 20 μg/mL UPK3A 65-84 for 48 hours. The supernatants of these cells were collected for cytokine analysis.

Cytokine analysis

The type and effectiveness of the immune response were assessed by murine sandwich ELISA measuring the IFN-γ (Peprotech, 900-K98), IL-2 (Peprotech, 900-K108), IL-4 (Peprotech, 900-K49), IL-5 (Peprotech, 900-K406), IL-17 (Peprotech, 900-K392) and IL-10 (Peprotech, 900-K53) with the appropriate antibody pairs and standards. The culture medium of cells isolated from the spleens of mice, which was collected five weeks after the final immunization, was used for the cytokine analysis after the cells had been cultured in 24-well, flat-bottomed plates with 20 μg/mL UPK3A 65-84 for 48 hours. The method was performed according to manufacturer's instructions. Briefly, 100 μL of capture antibody (1 μg/mL) were added to the wells, which were then incubated overnight at room temperature (RT). The wells were washed four times using 300 μl of washing buffer. The wells were then blocked with 300 μL blocking buffer at RT for one hour. Recombinant cytokine standards were prepared at different concentrations and added to appropriate wells, along with the samples obtained from culture supernatants which were incubated for two hours at RT with the corresponding cytokine antibodies. After washing four times, 100 μL of biotinylated secondary antibody (0.5 μg/mL) were added ito each well followed by a two-hour incubation period at RT. Avidin-HRP (1:2000) was added, followed by an additional 30 min incubation. One hundred μL of ABTS substrate solution was added after the last wash and the plate was read at 405 nm on an ELISA reader (Readwell Touch-Robonic). Standard curves were established using known concentrations of each recombinant cytokine, and sample cytokine concentrations were determined from values within the linear part of the standard curve.

Flow cytometry

Five weeks after the final immunization, splenocytes were isolated and were seeded at a density of 5×10^6 cells per well in 24-well, flat-bottomed F plates (BD Biosciences) in a total volume of 2 mL/well in DMEM (Lonza) supplemented with 10% fetal bovine serum (Biochrom), 5% HEPES buffer (Lonza), 2% L-glutamine, and 1% penicillin/streptomycin (Biochrom). The splenocytes were activated with the addition of 10 μg/mL peptide *in vitro* for 72 h at 37°C in humidified air with 5% CO_2. After washing with PBS, the reactivated splenocytes were suspended in flow cytometer solution (FACS) (PBS + 0.1% sodium azide + 2% BSA) at a density of 5×10^6 cells/mL. The cell suspension was aliquoted having 1×10^6 cells/sample per condition and were stained with corresponding fluorochrome-labelled antibodies in a final volume of 100 μl FACS buffer (cell suspension + antibody mix). The cells were incubated for 30 min at 4°C in the dark. Cells were washed twice with FACS buffer. After the final washing step, 500 μL of 1% paraformaldehyde (in PBS + 0,1% sodium azide) was added and cells were analyzed using a BD FACSCanto™ II (BD Biosciences) flow cytometry device. The following fluorochrome-labeled monoclonal antibodies were used; anti-CD3 PE-Cy5.5 for T-lymphocytes, anti-CD19 PE for B lymphocytes, anti-CD4 FITC for T-helper lymphocytes, anti-CD8 PE for cytotoxic T lymphocytes, anti-CD11c FITC for dendritic cells (eBiosciences).

Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

Expression levels of mRNAs for TNF-α, NKRP1A, CD11b, CD11c, CD45, IFN-γ, IL-2, IL-4, IL-10, IL-17 were measured in cultured splenocytes of mice, five weeks after final immunization with KLH-conjugated UPK3A 65-84+CFA or CFA alone, or with KLH-conjugated UPK3A 65-84+CpG or CpG alone. Total RNA was extracted from the spleen cells cultured for 72 h with activation by 50 μg/mL peptide using TRIzol reagent (Invitrogen). cDNA was synthesized from RNA using a GoScript™ Reverse Transcription System kit (Promega, A5001) according to the manufacturer's instructions. The primer pairs for qRT-PCR were designed using the online Universal Probe Library Assay Design Center (Roche, Mannheim, Germany). The sequences of the primers used are listed in *table 1*. qRT-PCR was performed using GoTaq qPCR Master Mix kit (Promega) with the LightCycler® 480 Real-Time PCR System (ROCHE). The gene expression levels were normalized to expression of the housekeeping gene β-actin and relative to the average level in mice immunized with CFA or CpG were calcu-

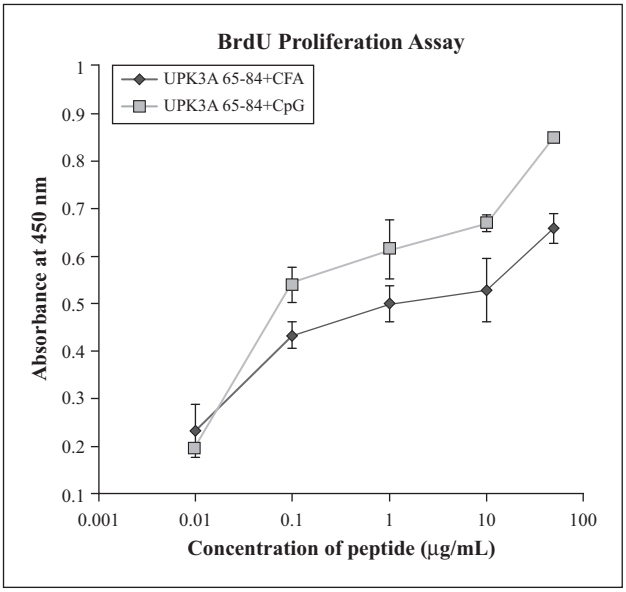

Figure 1
Antigen-specific recall proliferative responses of lymph node cells. Results of BrdU assays of BALB/c LNC incubated with serial dilutions of UPK3A 65–84 (filled shapes) are expressed as the mean optical density (O.D). The proliferative response to peptide was higher in LNCs from mice immunized with KLH-conjugated UPK3A 65–84 in CpG. The bar represents standard deviation (n = 3 per group).

lated using the comparative CT method, after confirming that the mean levels of beta-actin mRNA did not differ significantly between the immunized mice and CFA- or CpG-injected mice. Data are represented as mean ± SD of triplicate experiments.

Statistical analysis

The unpaired, two-tailed Student's *t*-test was used to analyze differences between the stimulated KLH-conjugated UPK3A 65-84 with CFA or CpG and unstimulated KLH-conjugated UPK3A 65-84 with CFA or CpG in cytokine ELISA analysis. qRT-PCR data analysis was performed by one way ANOVA with Tukey's *post hoc* test. The statistical significance was considered as $P \leq 0.05$

RESULTS

Immunization with KLH-conjugated UPK3A with CpG as adjuvant results in better antigen-specific proliferative responses than KLH-with CFA.

Immunization by injection of KLH-conjugated UPK3A 65-84 into female BALB/c mice with either CFA or CpG as adjuvant, results in high immunogenicity, as shown by recall proliferation assays of lymph node cells (LNC) (*figure 1*). The proliferative response to the peptide was compared in LNC from mice immunized with CFA or CpG as adjuvant. LNC obtained from mice, 10 days after immunization with UPK3A 65–84+CFA (10-day-primed LNC) and UPK3A 65–84+CpG (10-day-primed LNC) had proliferated in response to UPK3A 65–84. The proliferative response to serial dilutions of the peptide was higher in the LNCs of mice immunized with KLH-conjugated UPK3A 65–84 with CpG.

Immunization with KLH-conjugated peptide with CpG as adjuvant resulted in higher production of IFN-γ, IL-2 and IL-17 than immunization with the antigen and CFA

Mice were immunized with 100 μg of UPK3A 65-84 with either CpG or CFA as adjuvant. The dose of CpG used was determined from the manufacturer's instructions. Five weeks later, splenocytes were harvested for ELISA and qRT-PCR. *Figures 2* and 3 show antigen-specific cytokine recall responses against UPK3A 65-84. ELISA of 48-hour supernatants from five-week-primed spleen cells, following exposure to KLH-conjugated peptide with CFA, or KLH-conjugated peptide with CpG, cultured with 20 μg of UPK3A 65–84, revealed a Th1-like cytokine recall response to UPK3A 65–84 characterized by enhanced expression of IFN-γ and IL-2, and limited production of Th2-associated IL-4, IL-5, IL-10. However, despite the production of substantial amounts of IL-2 and IFN-γ by both KLH-conjugated peptide+adjuvant immunizations, UPK3A 65-84 peptide administered with CpG actually showed higher amounts of IL-2 and IFN-γ upon antigen recall, compared with peptide plus CFA ($p = 0.0074$, $p = 0{,}0001$ respectively). When IL-17 responses were measured, KLH-conjugated peptide with CpG immunization resulted in much greater IL-17 responses compared with KLH-conjugated peptide with CFA immunization. These differences in IL-17 secretion in antigen-stimulated culture supernatants were highly statistically significant ($p = 0.003$). Moreover, the splenocytes of mice injected with KLH-conjugated UPK3A 65-84 peptide and CpG, stimulated with UPK3A 65-84 for 48 hours, showed much more IL-10 expression compared with peptide plus CFA ($P>0.05$). The average antigenic response for each cytokine is also shown in *figure 2*.

qRT-PCR was performed on the samples of cultured spleen cells for 72h with activation by 50 μg/mL peptide, five weeks after immunization with KLH-conjugated UPK3A 65-84+CFA or CFA alone, or with KLH-conjugated UPK3A 65-84+CpG or CpG alone. Highly statistically significant cytokine gene expression for IL-2, TNF-α and IFN-γ ($p = 0.022$ * $p<0.05$, $p = 0.001$ ** $p<0.01$, $p = 0.036$* $p<0.05$, respectively) was observed following immunization with KLH-conjugated UPK3A 65-84 peptide with CpG as adjuvant, compared to KLH-conjugated UPK3A 65-84 peptide with CFA (*figure 3*). Gene expression levels for IL-4, IL-5 and IL-10 ($P>0.05$) were not statistically significantly differentbetween the groups (*figure 3*).

Immunization with KLH-conjugated peptide with CpG as adjuvant resulted in greater $CD4^+$ T cell, $CD8^+$ T cell, $CD45^+$ cell, NK cell and $CD11b^+$ cell responses compared to immunization with KLH-conjugated peptide with CFA

The populations of CD4-, CD8-, CD19-, and CD11c-positive cells were analyzed by flow cytometry using anti-CD3 Pecy5.5, anti-CD19 PE, anti-CD4 FITC, anti-CD8 PE, CD11c FITC in splenocytes from mice immunized with KLH-conjugated UPK3A 65-84+CFA or with CFA alone, or with KLH-conjugated UPK3A 65-84+CpG or with CpG alone, cultured for 72h and stimulated with 10 μg/mL peptide *in vitro*. The popu-

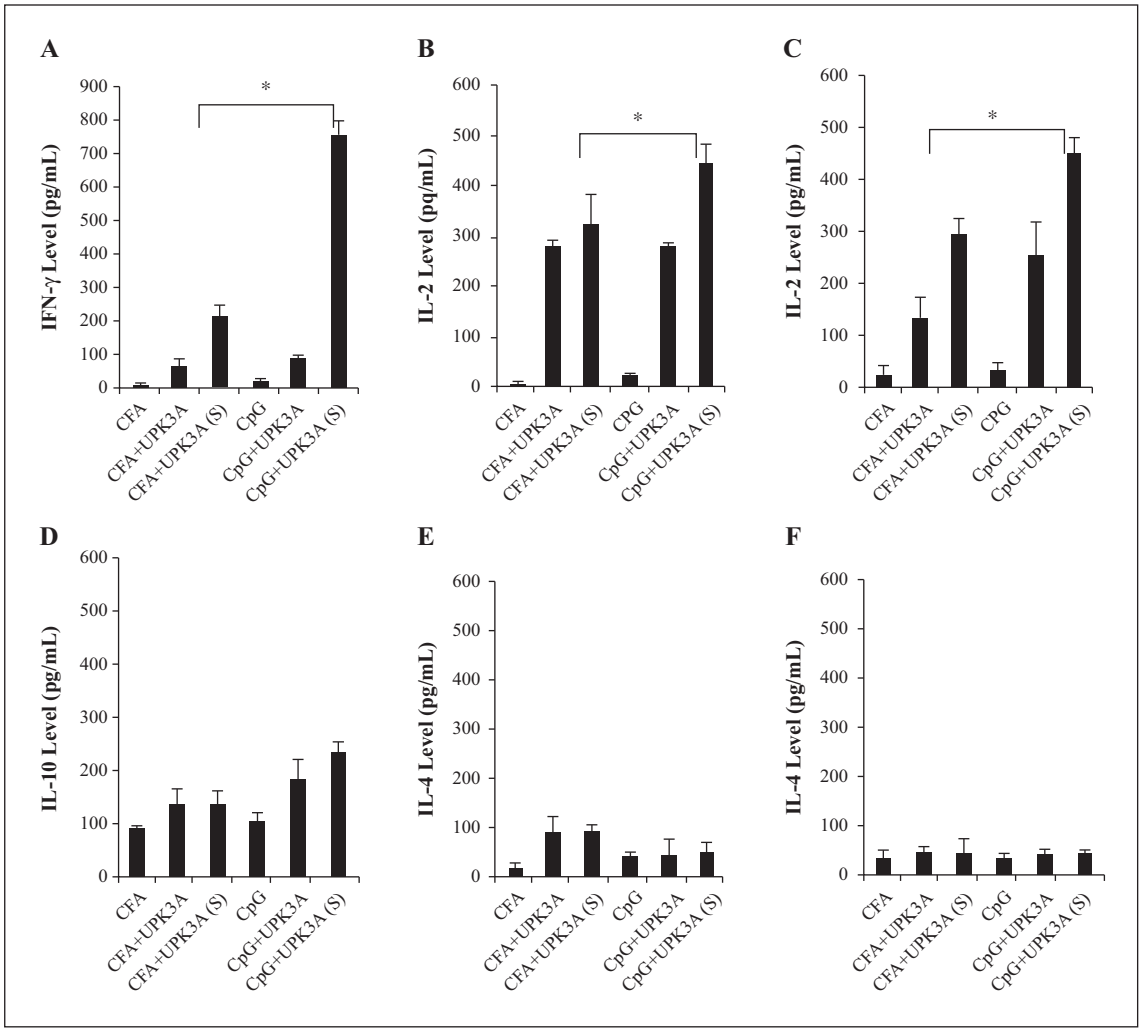

Figure 2

ELISA of cytokines. The ELISA analysis of supernatants of splenocytes from immunized mice, five weeks after final immunization, demonstrating a proinflammatory type-1 response to UPK3A 65–84, with high production of IFN-γ **(A)** and IL-2 **(B),** but limited production of Th2-associated IL-4 **(E)** and IL-5 **(F)**. **A-B)**-The recall immune response of mice immunized with KLH-conjugated peptide and CpG was significantly higher in terms of IFN-γ and IL-2 levels than the group immunized with antigen and CFA (n = 5 per group; *p = 0.0001 by unpaired t tests of UPK3A 65–84+CpG (stimulated) *versus* UPK3A 65–84+CFA (stimulated) ; * p = 0.007 by unpaired t tests of UPK3A 65–84+CpG (stimulated) *versus* UPK3A 65–84+CFA (stimulated), respectively). **C)** A high level of IL-17 secretion was observed in both the antigen+CFA- and CpG-immunized groups, but the level of IL-17 was highly statistically significant in the stimulated culture supernatant from mice immunized with antigen and CpG as compared to antigen and CFA (n = 5 per group; * p = 0.0033 by unpaired t tests of UPK3A 65–84+CpG (stimulated) versus UPK3A 65–84+CFA (stimulated)). **D)** The recall response of mice injected with KLH-conjugated UPK3A 65-84 peptide and CpG, and stimulated with UPK3A 65-84 for 48 hours, showed a much higher IL-10 production compared to antigen and CFA (p>0.05). Error bars indicate ± STD. (CFA+UPK3A = KLH-conjugated UPK3A 65-84 with CFA; CpG+UPK3A (S) = KLH-conjugated UPK3A 65-84 with CFA (Stimulated); CpG+UPK3A = KLH-conjugated UPK3A 65-84 with CpG; CpG+UPK3A (S) = KLH-conjugated UPK3A 65-84 with CpG (stimulated).

lation of CD4+ T cells was greater in the peptide with CpG -immunized group compared to the peptide-with CFA immunized group (*figure 4A*). While expansion of the $CD4^+$ T cell population after stimulation with peptide antigen was 2.4% for peptide and CFA compared to the unstimulated group, expansion of the $CD4^+$ T cell population after stimulation with peptide antigen was 3% for peptide and CpG compared to the unstimulated group. The $CD4^+$ T cell population of peptide and CFA after stimulation was 6.4% higher than the group receiving only CFA-(*figure 4A*). For the group with peptide and CpG, after stimulation, there were 8.6% more $CD4^+$ T cells than in the group receiving CpG alone (*figure 4B*).

The response of the CD8+ T cells was different between the groups immunized with CFA or CpG. There was a 27.5% decrease in CD8+ T cells in the UPK3A 65-84 with CFA group compared to the group receiving CFA alone-, and there was 12.8% decrease in $CD8^+$ T cells in the antigen-stimulated group compared to the unstimulated group (*figure 5A*). On the other hand, expansion of the $CD8^+$ T cell population after stimulation with antigen in the UPK3A 65-84 with CpG immunized group was 10.5% higher than the group receiving only CFA (*figure 5B*). Moreover, the increase in the $CD8^+$ T cell population occurred in the UPK3A 65-84+CpG group when stimulated *in vitro* with peptide antigen.

Analysis of the B cell population using flow cytometry revealed that this population was bigger the in group immunized with UPK3A 65-84+CFA than in the group immunized with UPK3A 65-84+CpG (*figures 6A, B*). While the B cell population was 7.2% bigger in the

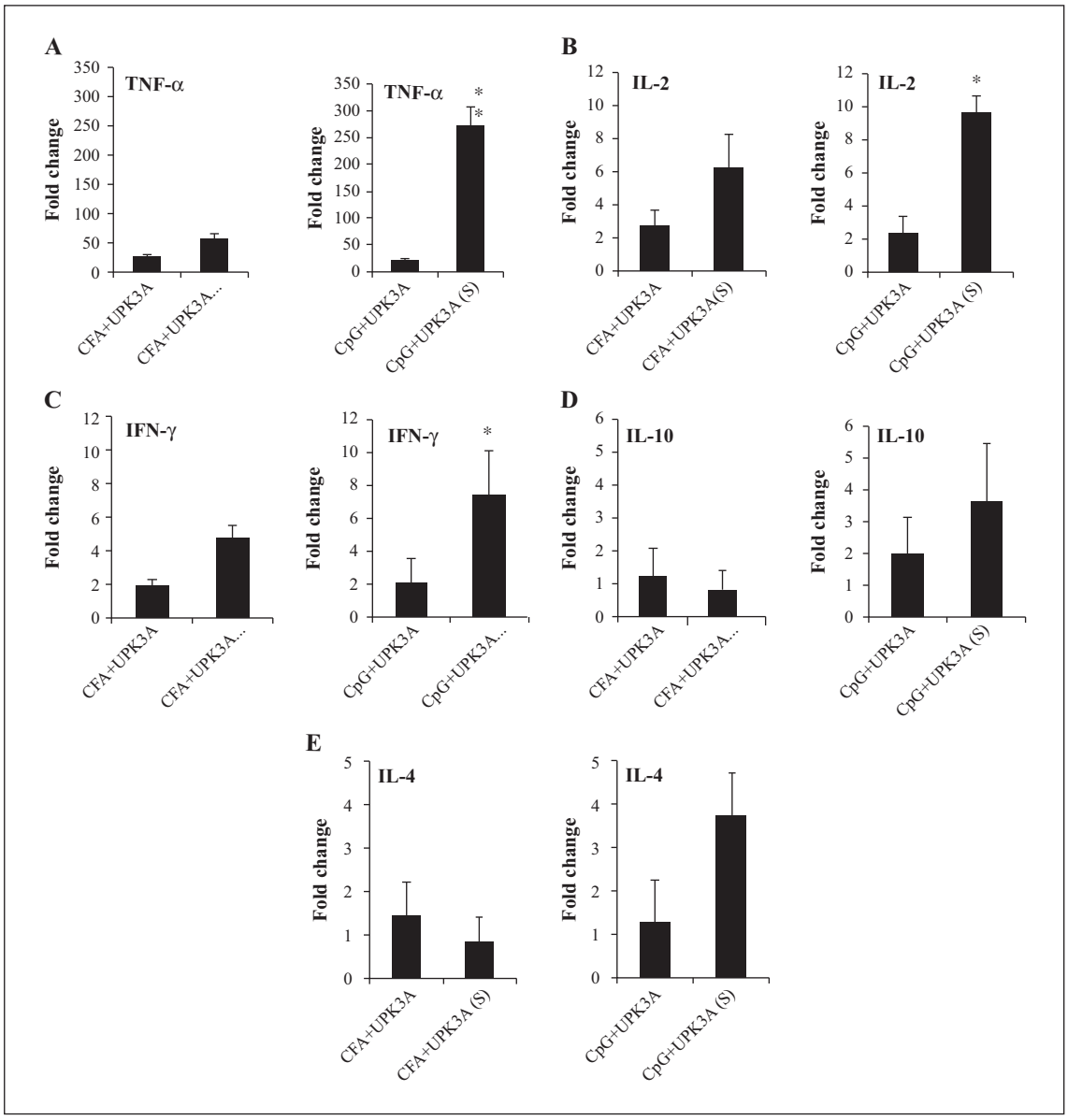

Figure 3

The cytokine gene expression analysis by qRT-PCR of samples of cultured spleen cells. Gene expression levels were normalized to expression of the housekeeping gene β-actin and relative fold-change to the average level with CFA and with CpG. The highly statistically significant cytokine gene expression of TNF-α **(A)**, IL-2 **(B)** and IFN-γ **(C)** ($p = 0.001$ ** $p<0.01$, $p = 0.022$ * $p<0.05$, $p = 0.036$* $p<0.05$, respectively) was observed following immunization with KLH-conjugated UPK3A 65-84 peptide with CpG as adjuvant compared to KLH-conjugated UPK3A 65-84 peptide and CFA. Cytokine gene expression levels of IL-10 ($P>0.05$) **(D)** and IL-4 ($P>0.05$) **(E)** were not significantly different between the groups. Error bars indicate plus STDEV of four mice per group, with triplicate assays for each mouse. *p for KLH-conjugated UPK3A 65-84 with CpG-immunized mice *versus* KLH-conjugated UPK3A 65-84 with CFA-immunized mice, by one way ANOVA with Tukey's *post hoc* test. (CFA+UPK3A = KLH-conjugated UPK3A 65-84 with CFA; CpG+UPK3A (S) = KLH-conjugated UPK3A 65-84 with CFA (stimulated); CpG+UPK3A = KLH-conjugated UPK3A 65-84 with CpG; CpG+UPK3A (S) = KLH-conjugated UPK3A 65-84 with CpG (stimulated).

group immunized with UPK3A 65-84+CpG than the group receiving CpG alone, this cell population was 3.8% bigger in the group immunized with UPK3A 65-84+CFA than in the group receiving CFA alone (*figures 6A, B*). The difference in the number of B cells between the group immunized with UPK3A 65-84+CpG and the group injected with CpG alone was greater than the B cell increment seen between the UPK3A 65-84+CFA group and the group injected with CFA alone.

Analysis of the dendritic cell population with CD11c antibody in flow cytometry showed that immunization with UPK3A 65-84 with CFA caused an approximately 2-fold increase in the $CD11c^+$ dendritic cell population (UPK3A 65-84 with CFA: 4.8%, UPK3A 65-84 with CpG: 2.2), compared to immunization with UPK3A 65-84 and CpG (*figure 6C*). Moreover, the RT-PCR result showed that gene expression of CD11c ($P>0.05$) was no different between the groups (*figures 7A*).

qRT-PCR was performed on samples of cultured splenocytes for 72 h, with activation by 50 μg/mL peptide, five weeks after immunization with KLH-conjugated UPK3A 65-84+CFA or CFA alone, or with KLH-conjugated UPK3A 65-84+CpG or CpG alone (*figure 7*). The highly statistically significant immune cell marker gene expression of CD45 ($p = 0.02$ * $p<0.05$), NKRP1A ($p = 0.042$* $p<0.05$), $CD11b^+$ cells (0.001 ** $p<0.01$) was

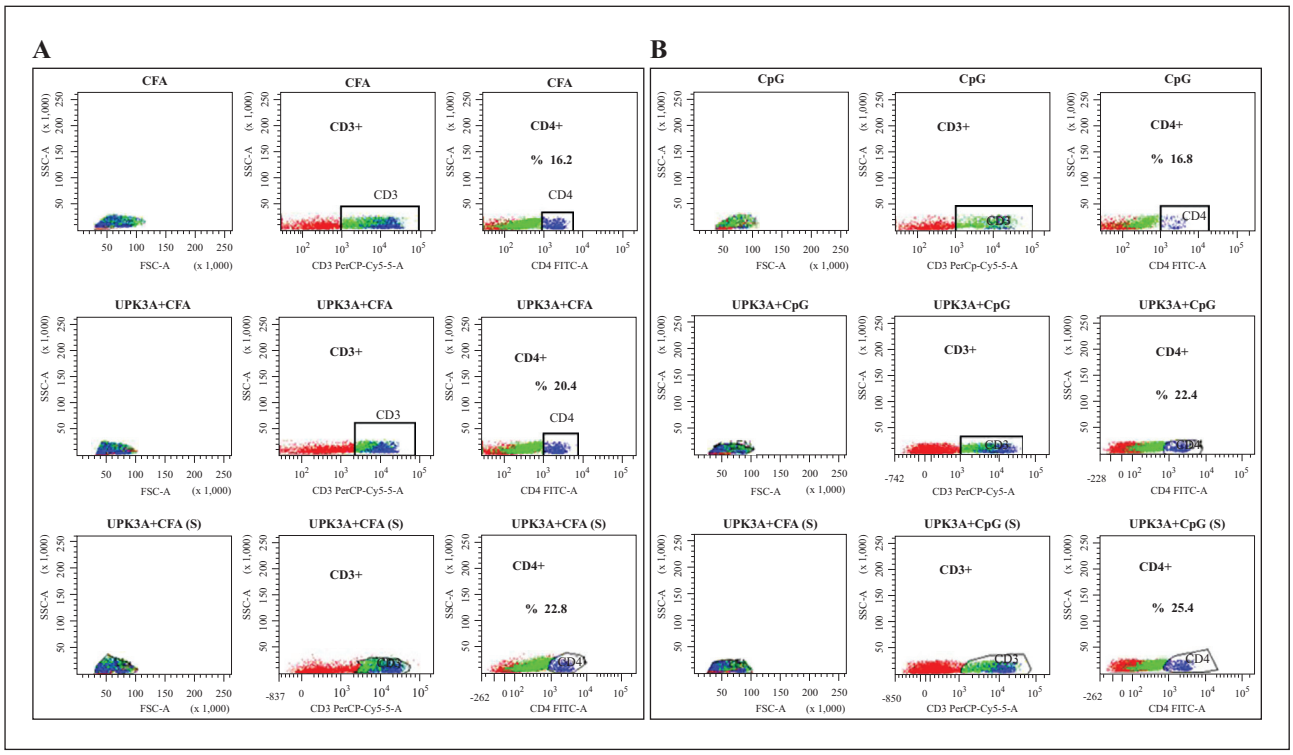

Figure 4

Flow cytometric detection of the CD4⁺ T cell response. Five weeks after immunization, cultured splenocytes were analyzed by flow cytometry using CD3PerCP-Cy5.5/CD4 FITC antibodies. The population of CD4⁺ T cells was higher in peptide-immunized group with CpG compared to peptide-immunized with CFA. A greater expansion of CD4⁺ T cells after stimulation with 10 μg/mL peptide was observed in the group immunized with UPK3A 65-84+ CpG than in the group immunized with UPK3A 65-84+CFA.

observed following immunization with KLH-conjugated UPK3A 65-84 peptide with CpG as adjuvant compared to KLH-conjugated UPK3A 65-84 peptide with CFA (*figures 7B-D*).

DISCUSSION

This study shows that CpG combined with KLH-conjugated peptide antigen (UPK3A 65-84) promotes a

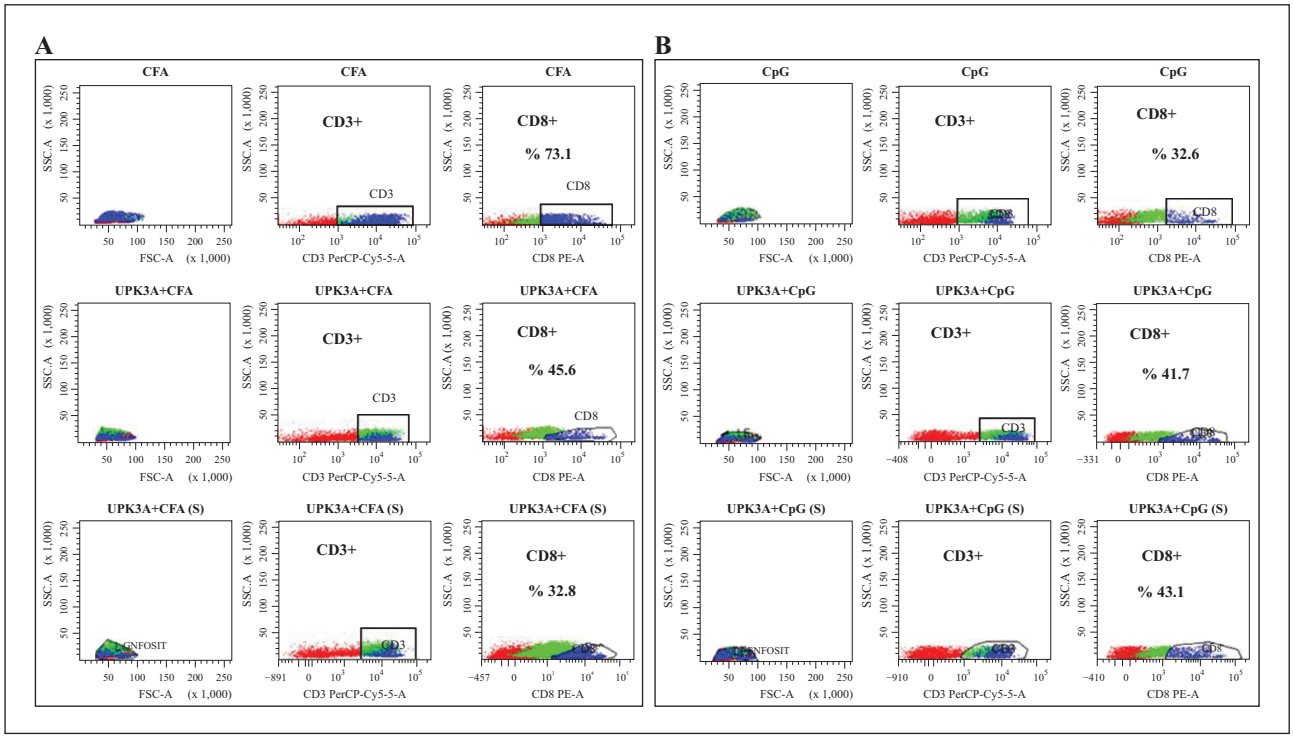

Figure 5

Flow cytometric detection of the CD8⁺ T cell response. Five weeks after immunization, cultured splenocytes were analyzed by flow cytometry using CD3PerCP-Cy5.5/CD8 PE antibodies. A higher expansion of CD8⁺ T cells after stimulation with 10 μg/mL peptide was observed in cells from mice immunized with UPK3A 65-84+ CpG than in the cells from mice immunized with UPK3A 65-84+CFA.

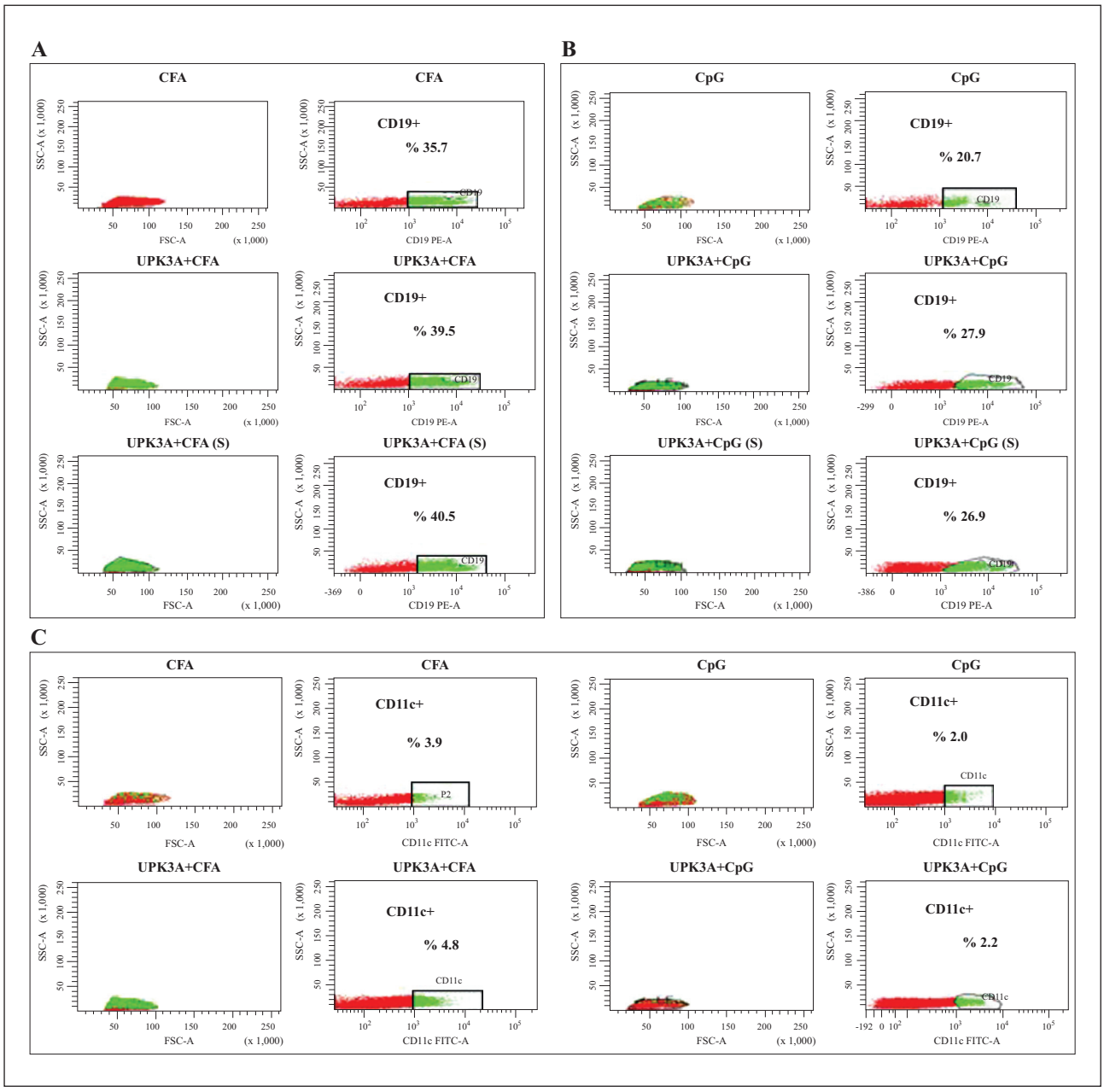

Figure 6

Flow cytometric detection of B cell and the dendritic cell response. Five weeks after immunization, cultured splenocytes were analyzed by flow cytometry using CD19 PE and CD11c FITC antibodies. The populations of CD19+ cells **(A, B)** and CD11c + cells **(C)** were higher in cells from mice immunized with UPK3A 65-84+CFA than the cells from mice immunized with UPK3A 65-84+CpG.

robust immune response via induction of higher levels of IL-2, IFN-γ, TNF-α, IL-17 and activation of more immune cells ($CD4^+$ T cell, $CD8^+$ T cells, NK cells CD11b, CD45) than the KLH-conjugated peptide antigen (UPK3A 65-84) with CFA.

More specific and effective treatments are needed for bladder cancer, as superficial bladder cancer treated with BCG immunotherapy inducing a non-specific immune response to the tumor has a high rate of recurrence and high toxicity. For this reason, many immunogenic treatment approaches, using recombinant cytokines such as IFN-γ, IL-2, IL-12 have begun to be tested in *in vivo* cancer models [2, 28, 29]. BCG treatment generates a Th1-type immunogenic response in bladder cancer [2, 30]. The release of a wide range of immune-stimulatory cytokines such as GM-CSF, IFN-γ, IL-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL-18, TNF, chemokines and eotoxin has been shown to occur in the bladder after the BCG vaccine administration [31]. In several studies, $CD4^+$ T cells, $CD8^+$ T cells and NK cells have been shown to be involved in the elimination of bladder cancer after BCG administration [32, 33].

$CD4^+$ T helper (Th1) cells lead to a long-lasting immune response [34]. Even though CD4+ T cells are generally more efficient in tumor destruction than $CD8^+$ cells [35], the optimum tumoricidal activity is typically observed when both $CD4^+$ and tumor $CD8^+$ T-cells are involved [36]. While the activated $CD8^+$ T cells directly target and kill the tumor cells, the activated $CD4^+$ T cells cooperate in the induction of $CD8^+$ T effector cells by initiating inflammation, and assisting B-cells to produce an anti-tumor response [37]. We propose that the involvement of

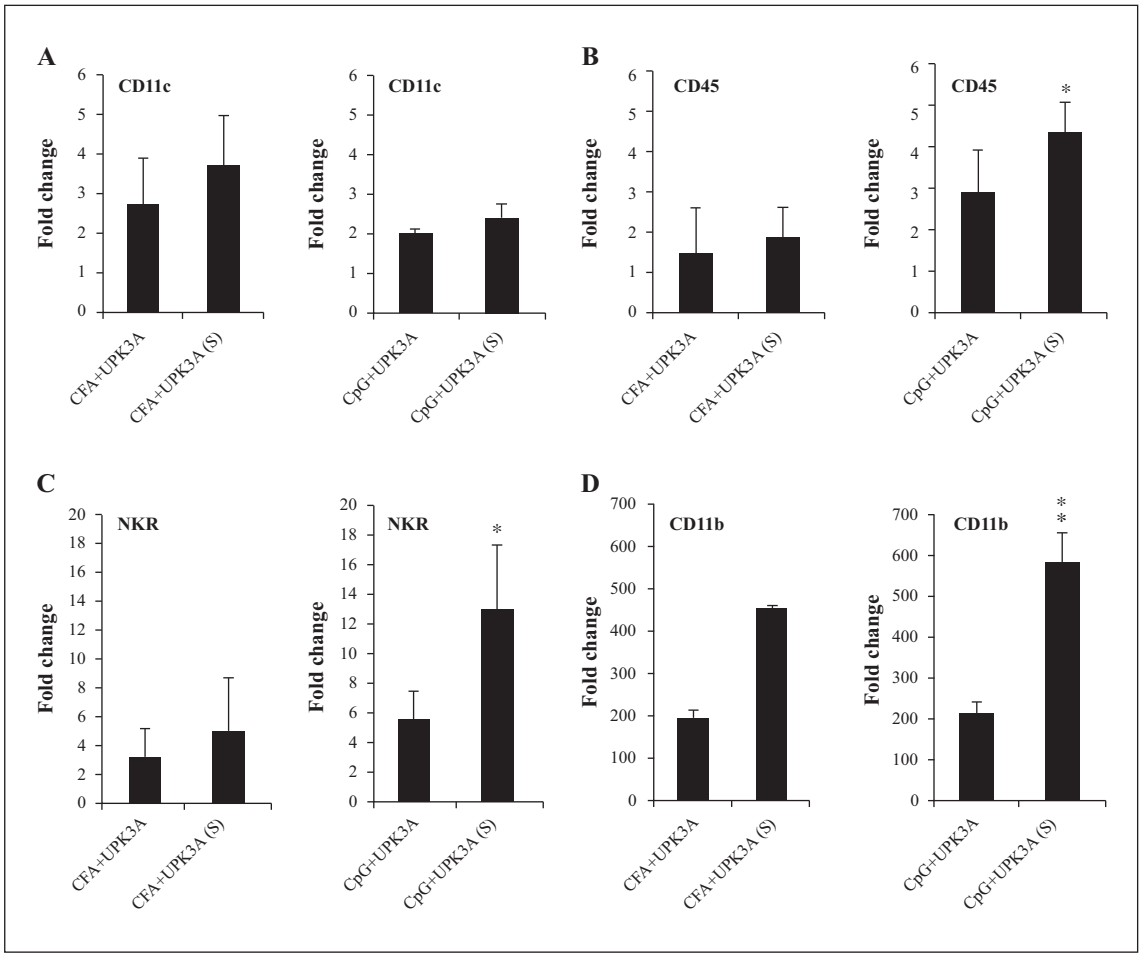

Figure 7

The gene expression of immune cell markers. The gene expression levels were normalized to expression of housekeeping gene β-actin and relative fold-change to the average level in CFA or in CpG mice. There was no difference in expression levels of CD11c (c) ($P>0.05$) between the groups **(A)**. A highly statistically significant immune cell marker gene expression of CD45 ($p = 0.02$ * $p<0.05$), NKRP1A ($p = 0.042$* $p<0.05$), $CD11b^+$ cells ($p = 0.001$** $p<0.01$) was observed in mice immunized with KLH-conjugated UPK3A 65-84 peptide with CpG as adjuvant, compared to KLH-conjugated UPK3A 65-84 peptide and CFA. (**B-D** respectively). There was no difference in expression levels of CD11c (c) ($P>0.05$) between the groups. Error bars indicate ± STD of four mice per group, with triplicate assays for each mouse. *p for KLH-conjugated UPK3A 65-84 with CpG-immunized mice *versus* KLH-conjugated UPK3A 65-84 with CFA-immunized mice, by one way ANOVA with Tukey's *post hoc* test. (CFA+UPK3A = KLH-conjugated UPK3A 65-84 and CFA; CpG+UPK3A (S) = KLH-conjugated UPK3A 65-84 and CFA (stimulated); CpG+UPK3A = KLH-conjugated UPK3A 65-84 with CpG; CpG+UPK3A (S) = KLH-conjugated UPK3A 65-84 with CpG (stimulated).

a $CD8^+$ T cell response could provide a more effective therapeutic result in bladder cancer therapy.

UPK3A is expressed in both normal bladder tissue and bladder cancer [38-40]. In many studies, the stable expression of UPK3A has also been shown in different human bladder cancer cell lines [41, 42]. It could be inferred that UPK3A protein expression in bladder cancer is highly preserved. Additionally, the proteins specific to bladder were used to develop experimental autoimmune cystitis models. The autoimmune experimental cystitis model in SWXJ mice has been developed to target Uroplakin proteins via an immune response to UPK2 (Uroplakin 2) self-antigens [5]. In our previous study, we developed peptide-specific, T cell-mediated autoimmune cystitis in the bladder of BALB/c mice using UPK3A 65-84 peptide (localized within the extracellular domain) injection with CFA specifically inducing a $CD4^+$ T cell-mediated immune response [13]. UPK3A is a well-chosen target for developing an autoimmune-mediated response against the antigen expressed in bladder tumor. The T-cell response and antibody formation against this highly expressed, bladder-specific peptide antigen, which can induce autoimmune cystitis, could be effective in the prevention and treatment of bladder cancer. In order to use this peptide derived from UPK3 protein in bladder cancer in vaccine studies, it is necessary to induce a stronger immune response. Our strategy is to boost the immune response achieved by UPK3A 65–84 peptide with well-chosen adjuvants.

An important phase in the immune response is the direction of T helper lymphocyte responses towards a type 1 response, especially Th1 (T helper 1). In this study, we selected two adjuvants: CFA and CpG oligonucleotides, which both trigger a Th1-type immune response. These two adjuvants are commonly used in cancer immunotherapy, particularly in preclinical models [5, 7, 20]. CpG has been recently used by researchers as a new and more effective adjuvant that generally stimulates Th1-type immune responses [22]. CpG used in cancer immunotherapy inhibited tumor growth significantly through the generation of antibodies, and evocation of $CD4^+$ and $CD8^+$ responses against the tumor antigen [23-26]. Moreover, the treatment

of mice with CpG was superior to BCG immunotherapy in the orthotopic bladder cancer model, and this treatment induced tumor-specific immunity by generating predominantly $CD4^+$ T-cells [27].

We administered repeated injections of the vaccines prepared with CFA or CpG, to increase clonal expansion of specific T cells and to ensure the attraction of the new clones to that site so they could increase the magnitude and diversity of the T cell response, as suggested in previous studies [34, 43]. In our study, immunization with peptide and CFA produced a marked expansion of CD4+ T cells, but only mild expansion of $CD8^+$ T cells. CpG was superior to CFA in the induction of the Th1 cytokine response, and in the activation of $CD4^+$ T cells, $CD8^+$ T cells, NK cells and B cells (*figures 2-6*).

Tumor cells can develop an escape mechanism against the antitumor immune response. While tumor-specific, cytotoxic T-lymphocytes and T helper cells are active in the early stages of tumor development, these cells lose their cytolytic function over time as the tumor grows [44, 45]. Cancer can also escape the anti-tumor immune response during the development of tumors due to the presence of increased immunosuppressive $CD25^+$ $CD4^+$ regulatory T (Treg) cells [46, 47]. A T regulatory (Tr1)-dominant immune profile has been observed as an increased level of the inhibitory cytokine IL-10, increased TGF-β expression, and the presence of regulatory T cells in patients with urothelial bladder carcinoma [48]. Therefore, conjugation of the peptide with an immunogenic carrier protein (KLH), and the use of effective adjuvants in the induction of an immune response against the antigen are important for mitigating cancer immune tolerance and preventing tumor escape from the immune response.

Our study showed that the immunization of mice with KLH-conjugated UPK3A 65-84 and CpG, induced a more effective immunization, with higher production of IL-2, IFN-γ, TNF-α, IL-17, and induction of more immune cells and immune cell markers ($CD4^+$ T cell, $CD8^+$ T cells, NK cells CD11b, CD45) than with the antigen and CFA. Levels of IL-10, IL-5, and IL-4 were found to be low in mice immunized with both CFA and CpG.

In this study, the expression of IL-17 was also significantly higher in mice immunized with antigen and CpG than antigen and CFA. Even though several controversial reports exist concerning the anti-tumor effect of IL-17, it has been suggested that Th17 autoimmunity could be used in anti-cancer immunotherapy via the recognition of tissue-specific antigens expressed both in tumor and normal tissue [49, 50].

Bladder-specific, UPK3A-derived peptide, with different adjuvants, would be a useful direction for future studies involving the evaluation of anti-cancer effects in preclinical models.

In conclusion, CpG as an adjuvant combined with KLH-conjugated peptide antigen (UPK3A 65-84) could be used in preclinical models of bladder cancer for the development of cancer immunotherapy strategies.

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