Quantum dot/pMHC multimers vs. phycoerythrin/pMHC tetramers for identification of HLA-A*0201-restricted pHBV core antigen$_{18-27}$-specific T cells

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Abstract. Detection of human leukocyte antigens-A2-restricted p-hepatitis B virus (HBV) core antigen-specific cytotoxic T lymphocytes (CTLs) is important in the study of HBV immunopathogenesis and vaccine design. Currently, major histocompatibility complex (MHC) class I/peptide-(p) MHCI tetramers are considered the optimal tools to detect antigen-specific CTLs. However, the MHC-tetramer technique also has certain drawbacks and is under continuous development. The quantum dot (QD) bioconjugates nanotechnology with its unique inorganic-biological properties has been developing fast. However, QD/pMHC multimers have seldom been used for the identification of the C_{18-27} epitope, which is important in HBV infection. QD/pMHC multimers were synthesized by metal-affinity coordination and an avidin-biotin system. In the present study they were characterized by transmission electron microscopy, dynamic light scattering and fluorescence spectrophotometry. C_{18-27}-specific CTLs were obtained by *ex vivo* expansion of $CD8^+$ T cells. Cultured CTLs were tested for the secretion level of interferon (IFN)-γ by ELISA and for cytotoxicity by lactate dehydrogenase release assay. Then, the performance of phycoerythrin (PE)/pMHC tetramers and QD/pMHC multimers were compared by flow cytometry. The synthesized QD/pMHC multimers dispersed well and their emission spectrum exhibited only slight differences compared with original QDs. C_{18-27}-specific CTLs not only secreted IFN-γ but also effectively targeted T2 cells pulsed with peptide $C_{18-27.}$ The frequencies of C_{18-27}-specific CTLs determined by QD/pMHC multimers were higher compared with PE/pMHC tetramers. The present results suggested that QD/pMHC multimers may be able to characterize greater numbers of C_{18-27}-specific CTLs with increased sensitivity compared to conventional strategies.

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Introduction

Chronic hepatitis B virus (HBV) infection is an extremely serious health problem worldwide with a number of ensuing complications including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (1,2). Statistical data indicate that ~400 million people are chronically infected with HBV (3). It has been reported that cellular immune responses serve an important role in preventing the deterioration of HBV infection (4). HBV-specific cytotoxic T lymphocytes (CTLs) are known to serve a critical role in cellular immune responses; efficient estimation of their numbers significantly improves the study of the viral clearance and disease pathogenesis (5-7). For the identification of HBV-specific CTLs, the human leukocyte antigen (HLA)-A*020-restricted p-HBV core antigen ($HBcAg_{18-27}$) epitope C_{18-27} is a perfect candidate. First, HLA-A2 is the most prevalent HLA allele globally so this epitope may be used for targeting virus-specific CTLs in almost half the population (8). Second, the therapeutic C_{18-27} peptide has been detected in the majority of HLA-A2.1 patients with HBV (9,10). Third, it has been reported that the presence of valine at the C-terminal of C_{18-27} peptide is associated with severe liver inflammation in Chinese patients with chronic HBV infection (11). Together, these reasons demonstrate that the characterization of C_{18-27}-specific T cells with high sensitivity is vital for the development of immunotherapeutic approaches to diseases caused by HBV.

A wide range of assays have been developed to measure T-cell immune responses (12) and among them peptide-major histocompatibility complex (pMHC) tetramers are the most common and standard methods (13). The fluorescence-labeled pMHC tetramer binds to a specific T-cell receptor (TCR) on the surface of a T cell using the nominal peptide imitating the corresponding antigenic epitope. This enables the detection of T cells by flow cytometry *ex vivo* (14). The pMHC tetramer technique is a powerful method to study T-cells, allowing direct and rapid visualization and quantification (15). It should be noted that the pMHC tetramers technique has its own drawbacks and remains under improvement. Phycoerythrin (PE) is the most commonly used organic fluorochrome for labeling tetramers (16). Its size is ~0.5-1 nm (17) meaning that normally just one avidin can be attached to a single PE organic fluorophore. The PE/pMHC tetramers usually contain four biotinylated pMHC monomers resulting in an avidin molecule has just four biotin-binding sites (18; as illustrated in Fig. 1A). Thus, they often fail to characterize CTLs where the interaction between pMHC and TCR is relatively weak.

The new emerging inorganic quantum dot-based multimers exhibit promising applications in cellular labeling and possess numerous advantages over conventional tetramers. Semiconductor nanocrystal QDs are spherical particles with diameters typically ranging between 2-20 nm, and consist of a semiconductor core (including CdSe and CdTe) alone or coated with a shell. The shell of the QD (e.g., ZnS) enhances the quantum yield and protects the core from oxidation (19). QD bioconjugates have been found to be 2,600-fold more resistant to photobleaching than PE (20). The narrow emission and broad excitation spectra from different QDs can be excited by a single light source (21). In addition, a single QD can be labeled with several proteins simultaneously. It has been reported that ~15-20 maltose binding proteins can be stably coated on each 6 nm diameter QD (22). Thus, a number of streptavidin molecules may be attached to one QD nanoparticle, leading to the QD/pMHC multimers carrying more pMHC monomers compared with standard pMHC tetramers (as illustrated in Fig. 1B). These QD/pMHC multimers offer a unique architecture for increasing the affinity of the TCR-pMHC interaction and may perform higher detection frequencies than PE/pMHC tetramers.

To date, a number of authors have made the use of QD/pMHC multimers for immunophenotyping (23-25). However, to the best of our knowledge, QD/pMHC multimers have not been used for the identification of the C_{18-27} epitope which serves an important role in the HBV infection. Thus, the present study used the QD/pMHC multimers technique to detect the *ex vivo* expansion of C_{18-27}-specific T cells by flow cytometry with a single 488-nm excitation light source. The aim of the current study was to examine whether QD/pMHC multimers can be used to stain C_{18-27}-specific T cells and if the technique were preferable to standard pMHC tetramers.

Materials and methods

Materials. The CdTe/CdS/ZnS core/shell/shell QDs were purchased from Beida Jubang Science & Technology Co. Ltd. (Beijing, China) and the streptavidin (SA) from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Corning Lymphocyte Serum-free medium was used for T cell culture and purchased from Zhao Sheng Co., Ltd. (Beijing, China). The transporters associated with antigen processing-deficient HLA-A2-positive T2 cell line was obtained from the Guangxi Key Laboratory of Biological Targeting Diagnosis and Therapy Research (Guangxi, China). The K562 cell line (cat no. CCL-243) was purchased from Xiangf Biotechnology Co., Ltd. (Shanghai, Beijing). Peptides SLYNTVATL (SL9) and FLPSDFFPSV (C_{18-27}) were synthesized by the Chinese Peptide Company (Hangzhou, China). Biotinylated pMHC monomers were from Beijing QuantoBio Biotechnology Co., Ltd. (Beijing, China). All chemicals used were of the highest purity available.

Synthesis of water soluble QD/pMHC multimers and PE/pMHC tetramers. QDs with emission maximum of 635 nm were used in the present study. QD-SA conjugates were prepared via metal-affinity interactions between a polyhistidine (His)-tag and Zn on the surface of the QDs. The His-tag SA (HSA) was prepared as previously described (24). Then, 10 μl of 25 μM HSA was incubated with 10 μl of 5 μM QDs in DMSO for 2 h at room temperature to yield QD-SA. The bioconjugates were purified by membrane filtration and washed with DMSO three times. Then, 10 μl QD-SA was added to 50 μl biotinylated pMHC monomers solution (0.1 mM) and stirred in the dark for 10 min; this step was repeated several times and the multimers obtained were purified using a 100 kDa membrane filter. PE/pMHC tetramers were obtained by a similar procedure but by adding PE-SA to biotinylated pMHC monomers solution.

Characterization of QDs and QD bioconjugates. The variety of QDs used in the present study was designated CdTe/CdS/ZnS core/shell/shell QDs. The surface properties of the QDs were characterized by transmission electron microscopy (TEM; H7650; JEOL, Ltd., Tokyo, Japan) and high-resolution TEM (JEM-2100F; JEOL, Ltd.). QD-bioconjugates were characterized by TEM and dynamic light scattering (DLS; Malvern Zetasizer; Malvern Instruments, Ltd., Malvern, UK). A fluorescence spectrophotometer (F-7000; Hitachi, Ltd., Tokyo, Japan) was used to characterize the fluorescence properties of the nanocrystals.

Ex vivo expansion of HBcAg18-27-specific T-cells. Peripheral blood mononuclear cells were isolated from 100 ml whole heparinized blood of healthy HLA-A*0201 volunteers by Ficoll/Hypaque density gradient centrifugation with 120 x g at 25˚C for 20 min (Sigma-Aldrich; Merck KGaA). HLA class I typing and A2 subtyping was performed by sequence-specific primer polymerase chain reaction (PCR). T2 cells in serum-free HEPES-buffered RPMI 1640 were pulsed with 40 μg/ml pHBV C_{18-27} (T2/C_{18-27}) at 37˚C for 4 h and irradiated by 200 Gy as stimulating cells. Effector cells were designated into two groups. One group was peripheral blood lymphocyte cells (PBLs), obtained by adherence to plastic for 1 h in complete RPMI-1640 medium (containing 2 mM L-glutamine, 100 U/ml each of penicillin and streptomycin, 1 mM sodium pyruvate, 1X MEM nonessential amino acids and 10% autologous serum). The other group was $CD8^+$ T cells immunobead-purified from PBLs. The effector cells were cocultured with stimulating cells at a ratio of 10:1 at 37˚C under 5% CO_2 and 20 IU/ml human interleukin-2 and

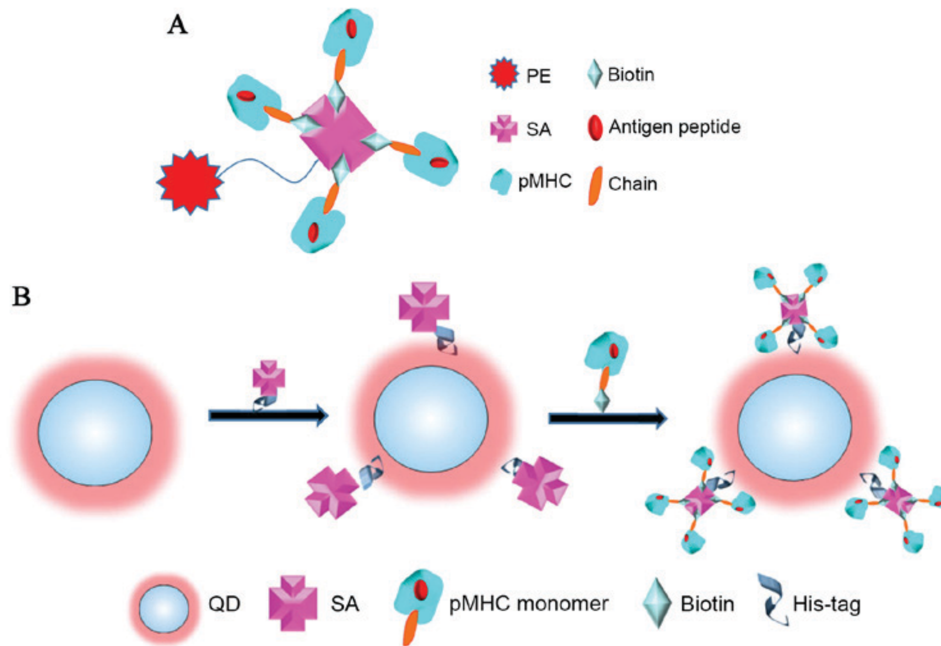

Figure 1. A schematic illustrating (A) the structure of PE/pMHC tetramers and (B) the synthesis process of QD/pMHC multimers. A PE/pMHC tetramer complex comprises one streptavidin molecule and four biotinylated pMHC monomers which consist of a light chain (β2-microglobulin), a heavy chain of HLA molecules and the antigen peptide of interest. The QD/pMHC multimers were generated through modification of QD with prepared His-tagged SA, then conjugation with biotinylated pMHC monomers to obtain a QD/pMHC multimer. PE, phycoerythrin; pMHC, peptide major histocompatibility complex; QD, quantum dot; His, polyhistidine; SA, streptavidin; HLA, human leukocyte antigen.

10 ng/ml human interleukin-7 were added into the coculture medium every 3 days. Lymphocytes were re-stimulated with C_{18-27}-pulsed irradiated T2 cells each week.

The study was approved by the Medical Ethics Committee of Guangxi Medical University (Nanning, China). Written informed consent was obtained from all subjects prior to enrolment in the present study.

Analysis of T2-MHC-I binding affinity. T2/C_{18-27} cells were incubated at 37˚C for 4 h in a 5% CO_2 atmosphere and irradiated at 200 Gy. Expression of MHC-I on T2/C_{18-27} and T2 cells subjected to the same procedures was determined by staining with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A2 monoclonal antibody (mAb) BB7.2. The fluorescence index (FI) was calculated as follows: FI=(mean FITC fluorescence with peptide C_{18-27}-mean FITC fluorescence without peptide)/(mean FITC fluorescence without peptide). Peptides with FI>1 were considered as high-affinity epitopes.

Interferon (IFN)-γ detection assay. To ensure that the T cells were activated, secretion of IFN-γ by the C_{18-27}-specific T cell cultures was evaluated by ELISA. OptEIA sets (BD Pharmingen, San Diego, CA, USA) were used to measure the concentrations of IFN-γ in supernatants of CTLs restimulated for 40 h with peptide-pulsed T2 cells. T2 were pulsed with peptides at concentrations of 40 µg/ml and used at the T cell: T2 cell ratio of 1:10. The IFN-γ in supernatants of unstimulated T and T2 cells served as negative control.

Peptide-specific CTL cytotoxicity assay. Cytotoxicity of C_{18-27}-specific CTLs was tested by a lactate dehydrogenase (LDH) release assay. The effector cells were C_{18-27}-specific T-cells from *ex vivo* culture. Target cells were T2 pulsed with FLPSDFFPSV (T2/C_{18-27}) and were cocultured at effector/target ratios of 10:1, 20:1 or 50:1 at 37˚C under 5% CO_2 for 4 h. K562 and T2 cells pulsed with SLYNTVATL (T2/SL9) were negative control groups. Each assay was performed in triplicate. Percent specific lysis=[(experimental release-spontaneous release)/(maximum release-spontaneous release)] x 100.

Characterization of peptide-specific T-cells by flow cytometry. In order to avoid the interference of non-specific cells, the effector T cells were divided into two groups: Peripheral PBLs and purified $CD8^+$ T cells. Following two weeks *in vitro* culture, PBL and $CD8^+$ T cells were collected and simultaneously labeled with FITC-conjugated anti CD8 mAb and PE/tetramers or QD/multimers. Four-color flow cytometry analysis was performed on a Coulter Epics XL cytometer with a single 488-nm excitation light source (Beckman Coulter, Inc., Brea, CA, USA). The flow cytometry data were analyzed in real time using Expo32 ADC software version 1.1C (Beckman Coulter).

Quantum dot cytotoxicity assay. For the cytotoxicity experiment, the harvested PBLs were loaded into 96-well microtiter plates and incubated with quantum dot/pMHC multimers (concentrations ranging between 0 and 5 µM) for 24 h at 37˚C. Each well contained 5,000 cells and samples were performed in triplicate. Then, 10 µl of the Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) solution was added to each well and the cells were incubated for another 2 h at 37˚C with 5% CO_2. The absorbance of samples was measured at 450 nm

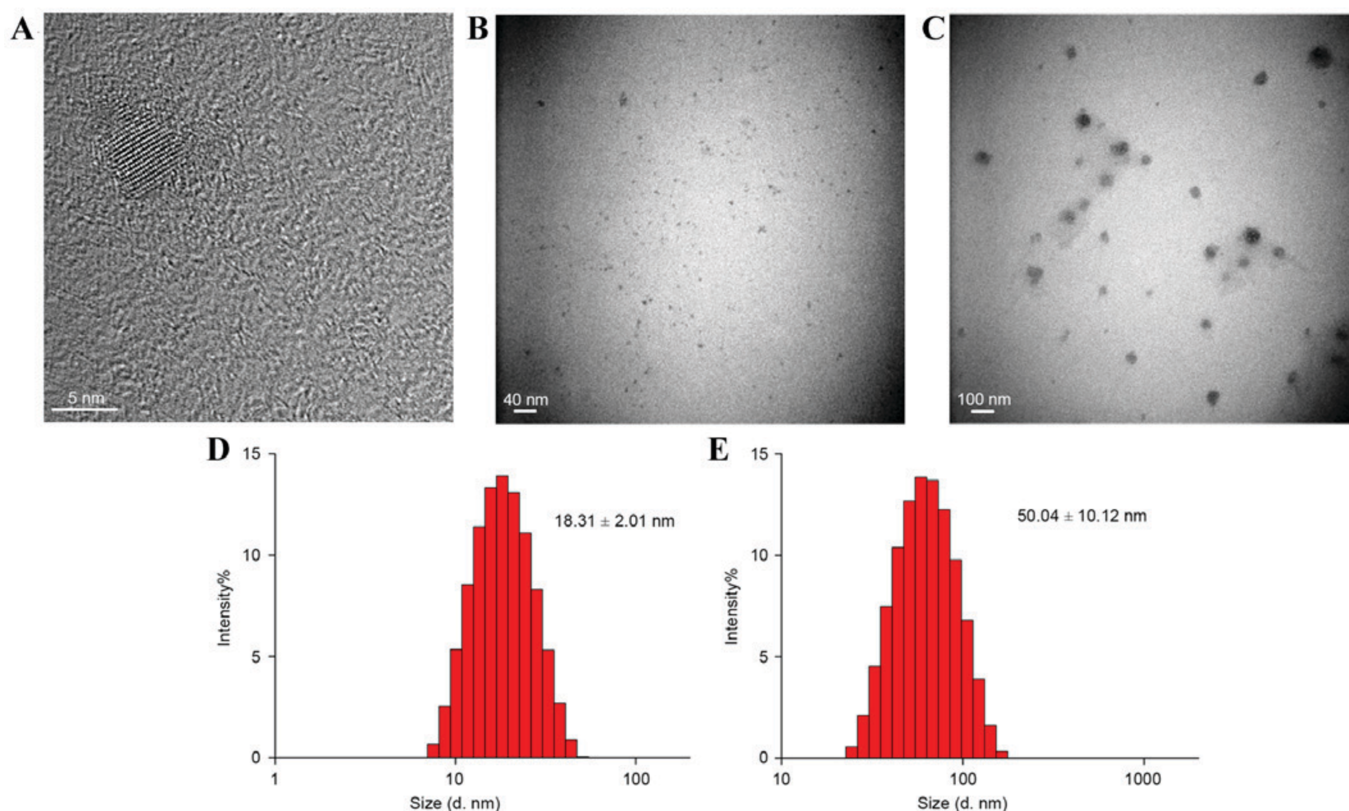

Figure 2. Sizes and dispersion of nanoparticles. High-resolution TEM images of (A) QD-625, (B) QD-625-SA and (C) QD/pMHC multimers. Hydrodynamic diameters of (D) QD-SA and (E) QD/pMHC multimers. TEM, transmission electron microscopy; QD, quantum dot; pMHC, peptide major histocompatibility complex; SA, streptavidin.

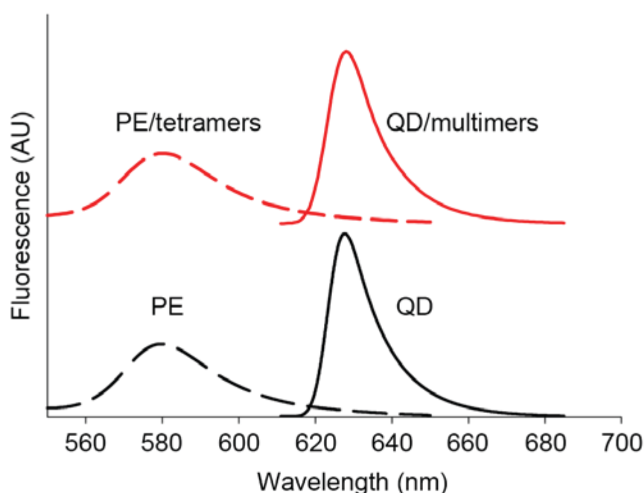

Figure 3. The fluorescence emission curve of original QD, PE and multimers. QD, quantum dot; PE, phycoerythrin; AU, arbitrary units.

by UV-vis with a Multiscan GO (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The number of cells was proportional to the optical density (OD) value and non-treated group was expressed as 100%.

Statistical analysis. Data from the experimental and control groups were analysed using one-way analysis of variance (ANOVA) followed by post hoc least significant differences, Student-Neuman-Keuls or Bonferroni tests for multiple comparisons. Data from the CCK-8 experiment were analysed using two-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference. Data are reported as the mean ± standard deviation. Statistical comparisons were performed using GraphPad Prism software version 6.0 (GraphPad Software,. Inc., La Jolla, CA, USA).

Results

Characterization of QDs and QD bioconjugates. TEM and DLS were used for the characterization of QDs, QD-SA and QD/pMHC multimers (Fig. 2). Results demonstrated that the diameter of QDs, QD-SA and QD/pMHC multimers were ~6.0, 18.31±2.01 and 50.04±10.12 nm, respectively (Fig. 2). The polydispersity index of all was <0.5. The fluorescence emission curve of original QD, PE and multimers (Fig. 3) showed that the fluorescence properties of both QD and PE had not been influenced by the surface modification process. These results indicated that the QDs and QD bioconjugate nanoparticles were well-dispersed and that the polymers or protein coated on QDs did not change the surface properties of the CdTe/CdS/ZnS nanocrystals.

Identification of stable HLA-1 expression in T2 pulsed with antigen peptide. To ensure that MHC-I molecules expressed stably on T2 cells, expression of HLA-A0201 on the surface of T2 cell membranes was examined by staining with mAb

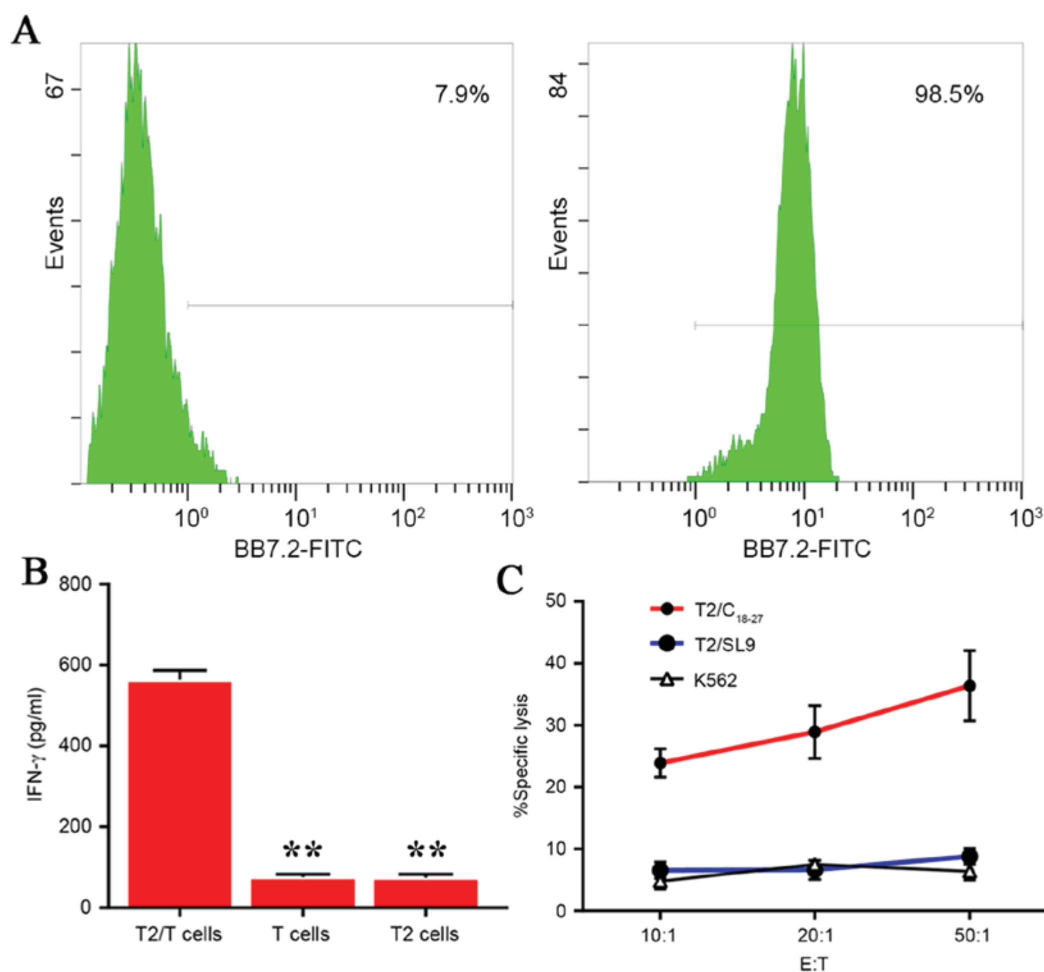

Figure 4. Three experiments were designed for the generation of CTLs. (A) The expression of HLA-I molecules by T2 cells untreated and T2/$C_{18\text{-}27}$ cells. (B) Resulting CTLs were tested for IFN-γ release using an ELISA assay and (C) $C_{18\text{-}27}$-specific lysis using LDH release assay. **P<0.01 vs. T2/T cells. CTLs, cytotoxic T lymphocytes; HLA, human leukocyte antigen; FITC, fluorescein isothiocyanate; IFN, interferon; LDH, lactate dehydrogenase.

FITC-BB7.2. As presented in Fig. 4A, a flow cytometry assay demonstrated that T2/$C_{18\text{-}27}$ cells expressed HLA-A0201 with markedly higher frequencies (98.5%) compared with T2 cells treated in similar conditions but without pulsing with antigen peptide, FI>1 (7.9%).

IFN-γ responses of peptide-specific T-cells from in vitro expansion. In order to confirm that the T cells had been activated by the selected functional epitope peptide $C_{18\text{-}27}$, the secretion of IFN-γ in supernatants of CTLs was tested by ELISA. As demonstrated in Fig. 4B, the IFN-γ secretion from $C_{18\text{-}27}$-specific CTLs increased markedly following *ex vivo* coculture with T2/$C_{18\text{-}27}$ for three weeks. In contrast, the T cells or T2 cells alone released significantly less IFN-γ compared with the T2/T cell experimental group (P<0.01; Fig. 4B).

Cytotoxicity of peptide-specific CTLs. The cytotoxicity of the cultured CTLs was investigated by LDH release assay. The results demonstrated that cultured specific CTLs lysed the T2/$C_{18\text{-}27}$ cells while the negative control groups (K562 and T2/SL9 cells) did not induce a CTL response and spontaneous release was below 10% of the maximum release (P<0.01; Fig. 4C). These results indicated that $C_{18\text{-}27}$ CTLs had been induced successfully *ex vivo*.

Comparison of QD/pMHC multimers and PE/pMHC tetramers for the detection of peptide-specific T cells. To compare the staining of $C_{18\text{-}27}$-specific T cells with PE/pMHC tetramers and QD/pMHC multimers, SL9 multimers or tetramers were designed as negative control materials. It is notable in Fig. 5 that QD/pMHC multimers detected higher numbers of $C_{18\text{-}27}$-specific CTLs than PE/pMHC tetramers. The frequencies in negative control groups were significantly lower.

Cytotoxicity assessment of pMHCI-QD multimers. To evaluate whether QD toxicity was harmful to T cells, a CCK-8 kit was used. Cytotoxicity data demonstrated that the percentage of cell death upon incubation of T cells with QD/multimers was low (Fig. 6). However, no significant difference in cytotoxicity between QD/multimers and PE/tetramers was detected. It also demonstrated that QDs with shells or coatings minimized Cd^{2+} release from the particle surface and that the CdTe/CdS/ZnS core/shell/shell QDs can be applied in T cell experiments.

Discussion

For the biological synthesis of QD/pMHC multimers, it is important to choose an appropriate assay for solubilization

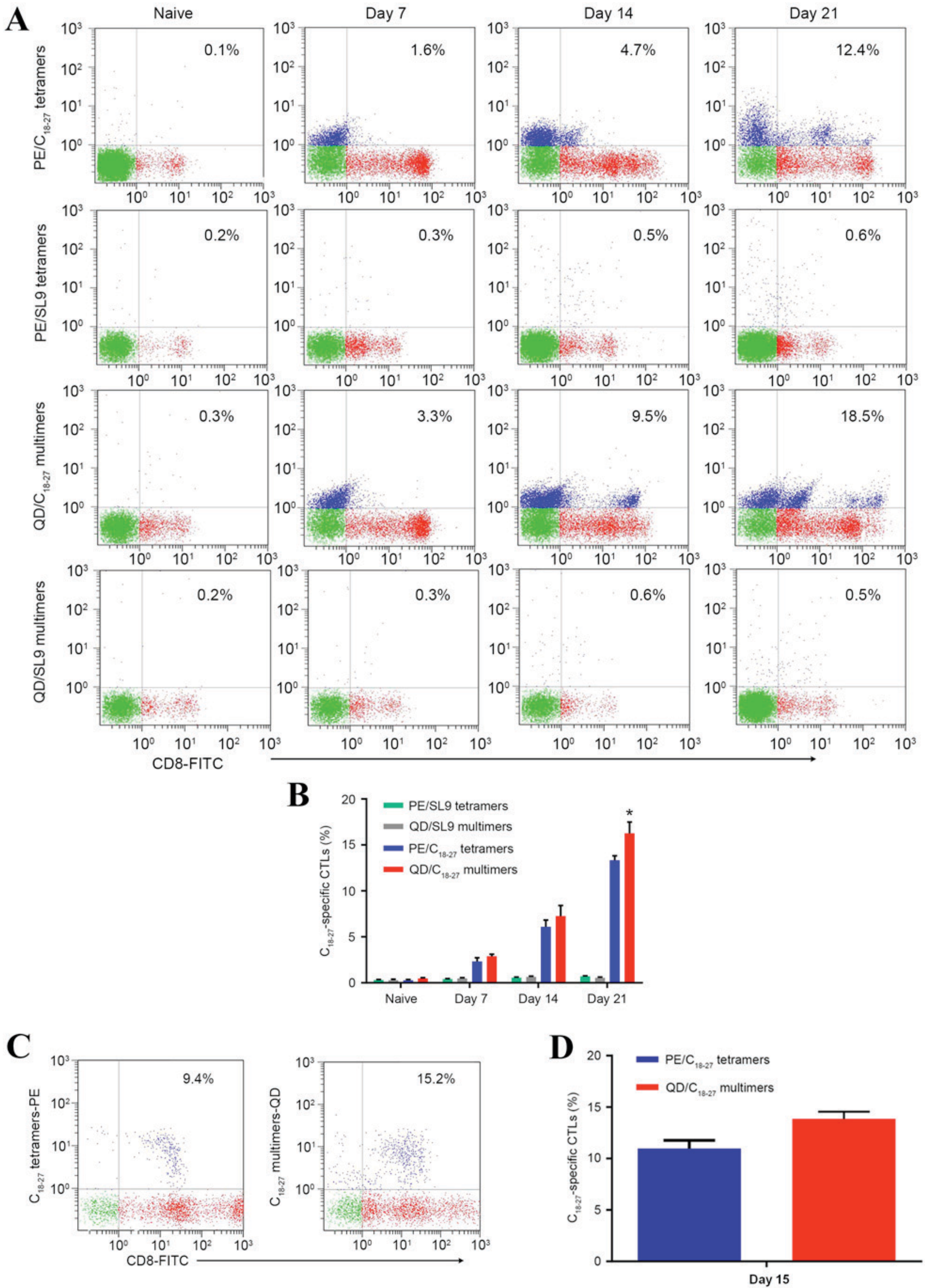

Figure 5. Identification of the frequencies of $HBcAg_{18-27}$ CTLs by QD/pMHC multimers and PE/pMHC tetramers with flow cytometry. (A) Frequencies detected each week during *ex vivo* coculture of T2/C_{18-27} cells with PBLs and (B) statistical chart (n=3). (C) Frequencies detected during *ex vivo* coculture of T2/C_{18-27} cells with purified $CD8^+$ T cells for 15 days and (D) statistical chart (n=3). HBcAg, human leukocyte antigens-A2-restricted p-hepatitis B virus core antigen; CTLs, cytotoxic T lymphocytes; QD, quantum dot; pMHC, peptide major histocompatibility complex; PE, phycoerythrin; C_{18-27}, $HBcAg_{18-27}$ epitope; PBLs, blood lymphocyte cells; FITC, fluorescein isothiocyanate.

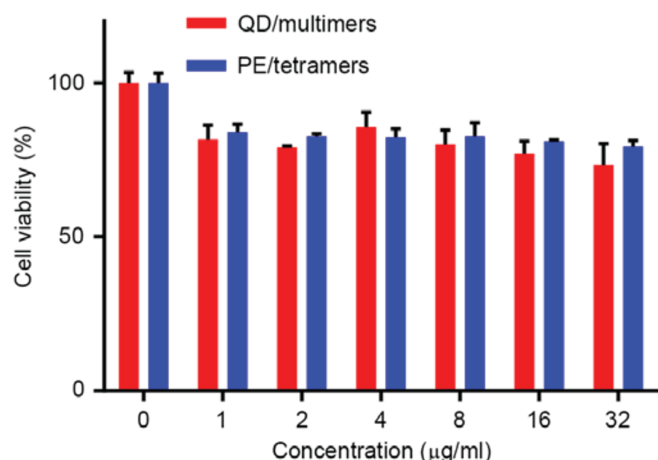

Figure 6. Evaluation of the cytotoxicity of QD/multimers compared with PE/tetramers by CCK-8 assay. QD, quantum dot; PE, phycoerythrin; CCK-8, Cell Counting Kit-8.

and stabilization of QD-bioconjugates in solutions (26). There are two main approaches to the surface modification of QDs: Covalent linkage and noncovalent binding (27-29). The covalent linkage assay usually results in QD-protein dispersions with a large aggregation and low quantum yield. The present study bound streptavidin to QDs via noncovalent metal-affinity interactions. Studies (28,30,31) have shown that in His-tag motifs to Zn atoms of QDs, the interaction (Zn^{2+}-His) is stronger than the majority of antibody bindings, so streptavidin was labeled with a His-tag to form high-affinity complexes with QDs. Then QD/pMHC multimers were obtained by conjugating monomeric pMHCI biotinylated-proteins to QD-SA. From the TEM images it can be observed that the QD/pMHC multimers nanoparticles are larger than QDs, so it is supposed that one single QD/pMHC multimer may contain several QDs and numerous pMHC monomers (Fig. 2C), although unforeseen aggregation did not appear. The resulting pellets demonstrated suitable stabilization (Fig. 2E) and the photoluminescence spectra did not demonstrate any noticeable difference in the peaks (Fig. 3), indicating that the surface modification did not induce any significant change in the structures of the QDs. One possible reason may be that the exposed charged groups and hydrophobic chains per polymer molecule in the QD-coating bioconjugates had changed little during the QD surface modification process (17).

QD toxicity has always been a great concern in biomedical studies (32). The cytotoxicity of QDs has been demonstrated to occur from released Cd^{2+} ions and can be greatly reduced by using QDs with shells or coatings (33). Cell viability is a commonly used assay to evaluate the toxicity of nanomaterials. In this study, CCK-8, which was considered to possess a improved sensitivity compared with the well-known MTT assay, was used to evaluate QD toxicity (34). The quantity of the formazan dye is directly proportional to the occurrence of living cells in this method. The relative viability of cells was obtained from the OD value at 450 nm wavelength. The CdTe/CdS/ZnS core/shell/shell QDs used in the present study did not show significant cytotoxicity (Fig. 6), suggesting that the CdS/ZnS coating of QDs greatly reduced the toxicity within the biological system.

For the coculture of T2/C_{18-27} and T cells, purified $CD8^+$ T cells were developed as one group of effector T cells in order to eliminate non-specificity influence of other cells in PBLs. To determine whether HLA-A*0201 expressed steadily on the surface of T2/C_{18-27} cells, the T2/C_{18-27} cells were stained with FITC-BB7.2 mAb. As demonstrated in Fig. 4A the peptide C_{18-27} bound to T2 with strong affinity. The secretion level of IFN-γ analyzed by ELISA assay demonstrated that C_{18-27} was markedly efficient at activating C_{18-27}-specific CTL responses (Fig. 4B). The cytotoxicity of peptide-specific CTLs detected by LDH release assay demonstrated that C_{18-27}-specific CTLs were induced successfully and the resulting CTLs had the ability to kill target cells (Fig. 4C). The experiments were designed to ensure that there was a sufficient number of functional C_{18-27}-specific T cells for parallel comparative analyses of T cell staining with PE/pMHC tetramers and QD/pMHC multimers.

During the expansion of C_{18-27}-specific CTL, PE/pMHC tetramers and QD/pMHC multimers were used to detect the frequencies of C_{18-27} specific CTLs. Fig. 5A and B demonstrate that QD/C_{18-27} multimers detected a higher number of T cells compared with PE/SL9 tetramers each week during *ex vivo* expansion. However, PE/SL9 tetramers exhibited a more stable detection result compared with QD/C_{18-27} multimers. When the effector T cells became purified $CD8^+$ T cells, the performance of the QD/C_{18-27} multimers was similar to PE/SL9 tetramers following *ex vivo* expansion for 10 days (data not shown). However, over 15 days the average CTL frequencies determined by QD/C_{18-27} multimers were markedly higher than PE/SL9 tetramers (Fig. 5C and D). The result may indicate that QD/C_{18-27} multimers exhibit higher detection efficiency compared with PE/SL9 tetramers when the frequency of C_{18-27}-specific CTL is relatively high. In brief, QD/pMHC multimers detected a higher number of T cells than PE/pMHC tetramers in the majority of cases and the QD/pMHC multimers technique can improve detection sensitivity.

Since Altman *et al* (18) first constructed MHC-tetramers for tagging T cells in 1996, MHC-tetramers technique have rapidly become the gold standard for T-cell analysis of known antigen specificity (35). The fast-moving development of QD-bioconjugates is stimulating interfaces with nanotechnology as well. The present study compared QD/pMHC multimers and PE/pMHC tetramers for staining C_{18-27}-specific CTLs expanded *ex vivo* with a single 488 nm argon laser for the first time and the results indicated that QD/pMHC multimer performed better in the analysis of C_{18-27} specific CTLs, which may improve the understanding of T cell immune response induced by HBV. It may also serve an important role in the development of more effective vaccines.

Acknowledgements

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