Quantum Dots as a Unique Nanoscaffold to Mimic Membrane Receptor Clustering

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ABSTRACT

Clustering of immune receptors and their ligands play an important role in the regulation of various responses of immune cells. However, very few tools to evaluate the effect of receptor and ligand clustering on live immune cells are available. We propose here to model major histocompatibility complex (MHC) proteins clusters. Quantum dots (QD) were used as a scaffold to assemble peptide-MHC (pMHC) molecules, the natural ligands for T-cell antigen receptor (TCR), into highly ordered oligomers with coherent orientation of individual protein molecules. Using pMHC/QD conjugates as a unique tool, we have shown that self pMHC proteins productively interact with the surface of T cells in CD8-dependent manner and facilitates recognition of foreign (viral) pMHC ligands at very low densities. We have also shown that pMHC/QD serve as an elegant probe to visualize uptake of productively engaged TCR and could be used to probe the extent of TCR and CD8 co-receptor clustering on the surface of live T cells at various stages of T cell activation and differentiation.

Keywords: quantum dots, membrane receptor clustering, major histocompatibility complex (MHC) proteins, T cells, T-cell antigen receptor and CD8 co-receptor

1 INTRODUCTION

It is thought that cell surface receptors on immune cells are clustered and could form "protein islands" that regulate antigen recognition and downstream signaling. It has been reported that MHC clusters on the surface of antigen-presenting cells (APC) increase T cell sensitivity to low density of agonist peptide-MHC (pMHC) ligands [1; 2]. To model MHC patches we utilized fluorescent nanoparticles, quantum dots (QD), as a scaffold to assemble various immune receptors into highly ordered oligomers with coherent orientation of individual protein molecules [3]. High local concentration of ZnS that constitutes the shell of QD mediates strong binding of hexahystidyl (His_6)-tagged proteins to the QD surface and the formation of stable protein/QD conjugates. QD of various sizes could accommodate from 10 to 40 protein molecules with molecular weight of about 50 kD. This allows assembly of various immune receptors or their ligands at designated ratios, to vary the distances between assembled molecules, and to evaluate how the extent of molecular clustering of these proteins influences immune recognition and kinetics of downstream signaling in live immune cells. Previously, we exploited fluorescent nanocrystals covered with dihydrolipoic acid (DHLA) to produce soluble, aggregate-free QD [3]. DHLA-capped QD are highly negatively charged and have proved to be very stable at alkaline pH, but they are prone to aggregation at neutral pH that is close to physiological conditions [4].

Here we present data comparing two types of QD carrying pMHC proteins that serve as ligands for antigen-specific T-cell receptor (TCR). These QD are differed from each other by the nature of QD cap, namely, DHLA-capped QD and QD encapsulated in lipid micelle. Inclusion of NiNTA (nickel-nitriloacetic acid) functionalized lipids into the micelle composition allows capturing His_6-tagged proteins. Such QD, termed NiNTA-QD, appear to be more stable at neutral pH. Analysis of functional properties and the binding of both kinds of QD to live T cells demonstrate similar results. In addition, NiNTA-QD have a lower surface charge that is close to that of the cell membrane and accommodate a higher number of pMHC proteins per dot making these dots a more versatile tool to mimic cell surface pMHC patches.

2 RESULTS

2.1 The number of pMHC molecules attached to NiNTA-QD

We compared the number of pMHC proteins conjugated with NiNTA-QD and DHLA-QD using experimental approach and theoretical calculations (Table 1). While His-tagged protein molecules interact with Zn^{2+} ions of the DHLA-QD shell, NiNTA-QD bind His-tagged proteins through formation of coordinate bonds with Ni-NTA functional groups conjugated with the micelle outer surface. Core size of various QD has been reported [5]. The size of ZnS shell that usually contains 5 monolayers amounts to ~1.2 nm [5]. The size of NiNTA-QD mainly depends on the dimensions of micelle and has been found to be ~ 5-7.5 nm [6]. To measure the amount of the conjugated pMHC we used gel filtration chromatography. The QD were incubated with pMHC at different ratio and the mixture was loaded on the gel filtration column. The presence of an excess of the pMHC resulted in two peaks. The second peak defined the amount of unbound pMHC molecules. The obtained numbers of pMHC per dot were in a agreement with the numbers calculated from the geometrical size of QD and the

Table 1: Number pMHC per QD

QD	Core radius, nm	Core-shell radius, nm	Capped core-shell radius, nm	Calculated pMHC number	Experimental pMHC number
DHLA-QD(520)	1.2	2.4	3.2	10-15	10
DHLA-QD(620)	2.6	3.8	4.6	25-40	40
NiNTA-QD(620)	2.6	3.8	5-7.5	40-150	100

size of protein molecules (Table 1). The 2-fold change in the diameter of DHLA-QD resulted in a 4-fold increase in the number of conjugated pMHC molecules. Thus, DHLA-QD and NiNTA-QD with the same core size accommodated 40 and 100 pMHC molecules, correspondingly.

2.2 Interaction of pMHC/NiNTA-QD conjugates with TCR on live T cells

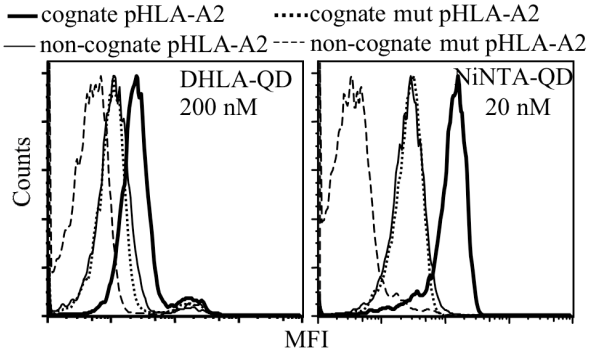

Figure 1: Binding of QD/pMHC conjugates to the surface of live CTL. The nature of pMHC complexes conjugated to the QD is indicated on the top of the figure.
Method: The QD/pMHC conjugates were self-assembled after mixing of DHLA-QD(520) or Ni-NTA-QD(620) and pMHC at ratios 1: 10 or 1:100 respectively. Human cytotoxic T lymphocytes (CTL) 68A62 were incubated with the QD conjugates at indicated concentrations for 30 min at room temperature and the samples were analyzed on a Coulter Flow Cytometer.

Our most recent data show that pMHC on the surface of QD have distinct spacing and positioning compared to pMHC molecules assembled into other oligomers (Anikeeva et al., in preparation), including Streptavidin-based tetramer or pMHC-Ig dimeric structures [7; 8]. Positioning of the pMHC molecules attached to QD apparently favors optimal multivalent engagement of the TCR and/or CD8 molecules on live CTL, explaining the binding of non-cognate conjugates and the cooperation between cognate and non-cognate pMHC ligands enhancing CTL responses. We compare the binding of cognate and non-cognate pMHC conjugates assembled on NiNTA-QD and DHLA-capped QD to the surface of live CTL. Fig. 1 shows that the cognate GL9-HLA-A2/QD and the non-cognate IV9-HLA-A2/QD conjugates bound equally strong to the surface of CER43 CTL regardless of the type of QD that is in marked contrast to other pMHC oligomers. The binding of the cognate conjugate with a single mutation in the non-polymorphic domain (A245V) of HLA-A2 protein (mutHLA-A2) that disrupts CD8-MHC interactions was decreased to a similar extent for the both QD types [3]. The binding of the non-cognate mutant conjugates (IV9-mutHLA-A2/QD) was indistinguishable from that of QD conjugated with irrelevant pDRB-1 (MHC class II loaded with peptide) proteins. Although the binding pattern of NiNTA-QD and DHLA-QD conjugates was the same, the binding of the NiNTA-QD conjugates was substantially stronger.

2.3 The pMHC/Ni-NTA-QD conjugates demonstrate higher potency than pMHC/DHLA-QD to stimulate T cells

We compared the ability of pMHC/QD conjugates produced with the DHLA-QD and the NiNTA-QD to trigger TCR-mediated signaling in concentration dependent manner. The assay is based on the analysis of Ca^{2+} flux in CTL labeled with Ca^{2+} fluorophore after stimulation with the strong agonist QD conjugates at various concentrations. As evident from Fig. 2, the CTL response induced by NiNTA-QD conjugates was approximately order of maghititude more sensitive then the response induced by DHLA-capped QD conjugates. This indicates, that changes in ligand valency influences the potency of QD/pMHC conjugate to stimulate CTL response.

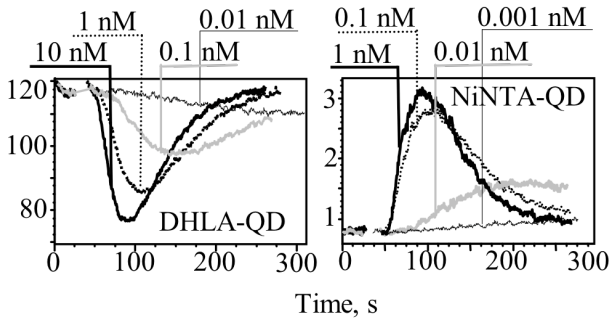

Figure 2: Time-dependent changes in intracellular calcium concentration in CER43 $CD8^+$ CTL induced by cognate pMHC/DHLA-QD(520) and pMHC/NiNTA-QD(620) conjugates at indicated concentrations.
Method: CTL were loaded with the dye sensitive to the increase of the intracellular Ca^{2+}. EGTA was added to the cell suspension to block Ca^{2+} in the extracellular medium. Freshly prepared QD conjugates were added to the CTL suspension at various concentrations and the samples were analyzed on a Coulter Flow Cytometer. The data collection was initiated as soon as possible following the background measurements.

2.4 Cooperation between cognate and non-cognate pMHC complexes in the induction of T cell responses as established with Ni-NTA-QD/pMHC conjugates

It is thought that pMHC clusters on the surface of virus-infected cells may contain pMHC complexes presenting both viral and self peptides. To mimic recognition of such clusters we prepared a series of pMHC/NiNTA-QD conjugates that carries cognate and non-cognate pMHC complexes with a progressively smaller average number of the cognate complexes per dot keeping the total number of the pMHC proteins constant, i.e., 100 per dot. Cognate and non-cognate pMHC complexes immobilized on the surface of these QDs efficiently cooperated in the binding to live CTL in CD8-dependent manner and in the induction of Ca^{2+} mobilization (Fig. 3), very similar to that previously described for DHLA-capped QD [3]. Because NiNTA-QD have a larger diameter and can accommodate up to 100 pMHC molecules per dot, these dots provide greater flexibility to manipulate proximity between cognate and non-cognate pMHC on the dots and to investigate the influence of separating distances on the cooperation between pMHC with various biological activities.

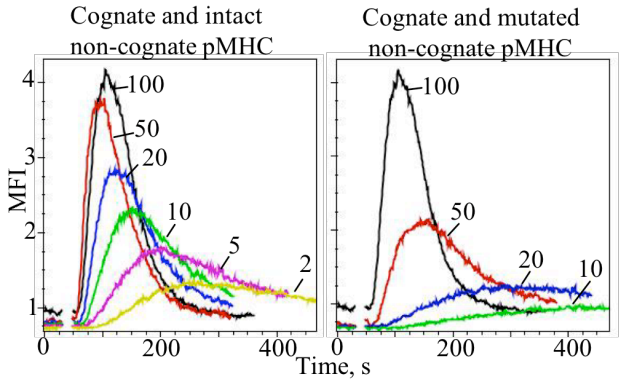

Figure 3: Cognate and non-cognate complexes displayed on the surface of NiNTA-QD at pre-determined ratios cooperate in the induction of Ca^{2+} mobilization in CER43 CTL in CD8-dependent manner. NiNTA-QD carried indicated numbers of the cognate and either intact (left) or mutated (right) non-cognate pHLA-A2 complexes. The total number of pMHC complexes per dot was kept constant.

2.5 Selective TCR-mediated uptake of pMHC/QD

To analyze the QD conjugates distribution at the cell surface and their retention in the intracellular compartment following TCR engagement, CTL were incubated with QD conjugates loaded with cognate or non-cognate pMHC. While CTL incubation with non-cognate conjugates produced a diffuse pattern of staining associated with the cell membrane, the incubation with cognate pMHC/QD for 10 minutes led to the QD clustering and retention within the intracellular compartment (Data not shown). Low pH treatment of the CTL removes the QD conjugates from the cell surface, but not those that had been internalized indicative of the QD uptake.

To provide evidence for rapid specific uptake of cognate pMHC/QD, we compared the extent of internalization of QD conjugates at 37°C and 4°C. Incubation at lower temperature is expected to strongly reduce TCR-mediated uptake. Fig. 4 shows that, indeed, the amount of cognate pMHC/QD internalized at 4°C is significantly lower than that at 37°C. Importantly, the internalization was evident even 10 minutes after QD/pMHC were added to CTL (data not shown), i.e. at the time when changes in the level of TCR and CD8 on the cell surface are not yet detectable. In addition, the experiment was performed in the medium without Ca^{2+} and Mg^{2+} to diminish contribution of adhesion molecules and to prevent uptake of extracellular Ca^{2+}. These data imply that specific TCR engagement is a necessary prerequisite driving initial pMHC/QD retention in intracellular compartments.

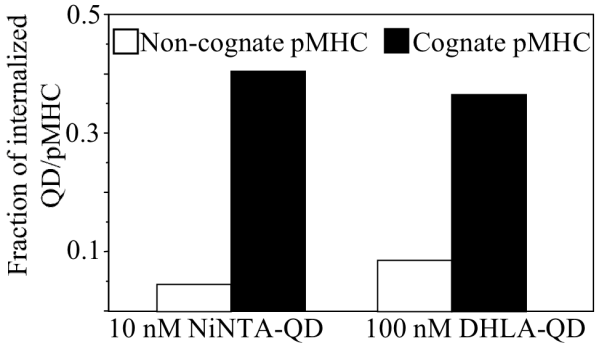

Figure 4: Quantification of the internalization of cognate pMHC/QD conjugates

Method: CER43 CTL were incubated with cognate or non-cognate pMHC/QD conjugates for 30 min at 37°C. After washing one half of each sample was treated with acid buffer, pH 2.5. Another half of the sample was used as a control. Acid treated cells were neutralized with an equal volume of PBS, pH 11.0. Samples were then analyzed by Flow Cytometry. The extent of pMHC/QD internalization by CTL was quantified as ratio of the mean fluorescence intensity of treated cells to untreated (control) cells.

3 DISCUSSION

Because the antigen-specific TCR uniquely marks every individual T cell and determines its reactivity, soluble oligomeric pMHC complexes are excellent tools to identify T cells with desired specificity and to stimulate T cell activation. Tetramers of pMHC have been successfully utilized for these purposes and have also proved to be useful for the induction of T cell activation as well for the stimulation of cell death in T cells with defined specificity [9]. In oligomers based on avidin or immunoglobulin

scaffolds the pMHC-pMHC distance is typically about 80 Å [10; 11] imposing constrains on their ability to be engaged in optimal multivalent interactions with both the T cell surface CD8 and the TCR that is necessary for efficient T cell activation. In fact, it has been shown that increasing the distance between pMHC subunits of a dimer above a certain threshold abolishes its effective binding to the T cell surface [12]. An optimal distance between pMHC arms in an pMHC dimer was estimated to be about 30 Å [11], i.e., substantially less than that in most oligomers. Our recent data show that pMHCs on the surface of QD have distinct spacing and orientation favors optimal multivalent engagement of the TCR and CD8 molecules on the T cell, enhancing binding avidity and cooperation between non-cognate and cognate pMHC that maximizes the sensitivity of T cell responses.

Our data show that the QD scaffold provides a unique opportunity to study self-pMHC contribution to the recognition of foreign pMHC regardless of the nature of outer shell. Moreover, the property of NiNTA-QD surface more closely mimics the cell membrane surface as compared to DHLA-QD. Because of the lipid nature of the outer cap, NiNTA-QDs are stable in solution without showing visible signs of aggregations for about one year. The nature of the lipid cap also determines the large NiNTA-QD radius and correspondently the high valency of proteins conjugated to NiNTA-QD. These characteristics of Ni-NTA-QD make them a valuable tool to investigation the role of ligand clustering recognizable by various immune receptors (for example, pMHC and ICAM-1) and the separating distances between the ligands and the receptors in controlling T cell responsiveness [13]. Changes in the composition of the clusters and the separating distances can function as a very sensitive mechanism regulating the immune response [14]. For instance, at the condition of inflammation, pro-inflammatory cytokines increase the level of expression of MHC and ICAM-1 molecules, facilitating clustering of these molecules that enhances stimulatory capacity of antigen-presenting cells [15]. The distances between TCR and co-receptors are also thought to be changing during differentiation and activation of T cells [16; 17]. These notions are consistent with well-documented heterogeneity of cell membrane and the presence of protein islands [18]. Changes of the protein distribution in these islands can serve as a fundamental mechanism regulating various activities of immune cells and other cells.

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