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Detection of Low-Avidity CD4+ T Cells Using Recombinant Artificial APC: Following the Antiovalbumin Immunological Response

Valérie I. Mallet-Designe,* Thomas Stratmann,* Dirk Homann,† Francis Carbone,‡ Michael B. A. Oldstone,† and Luc Teyton*2

Subtle differences oppose CD4+ to CD8+ T cell physiologies that lead to different arrays of effector functions. Interestingly, this dichotomy has also unexpected practical consequences such as the inefficacy of many MHC class II tetramers in detecting specific CD4+ T cells. As a means to study the CD4+ anti-OVA response in H-2d and H-2b genetic backgrounds, we developed I-A\b- and I-A\b-OVA recombinant MHC monomers and tetramers. We were able to show that in this particular system, despite normal biological activity, MHC class II tetramers failed to stain specific T cells. This failure was shown to be associated with a lack of cooperation between binding sites within the tetramer as measured by surface plasmon resonance. This limited cooperativeness translated into a low “functional avidity” and very transient binding of the tetramers to T cells. To overcome this biophysical barrier, recombinant artificial APC that display MHC molecules in a lipid bilayer were developed. The plasticity and size of the MHC-bearing fluorescent liposomes allowed binding to Ag-specific T cells and the detection of low numbers of anti-OVA T cells following immunization. The same liposomes were able, at 37°C, to induce the full reorganization of the T cell signaling molecules and the formation of an immunological synapse. Artificial APC will allow T cell detection and the dissection of the molecular events of T cell activation and will help us understand the fundamental differences between CD4+ and CD8+ T cells. The Journal of Immunology, 2003, 170: 123–131.

Selection and expansion of Ag-specific T and B cells follow infections, vaccinations, and autoimmune responses. As much as B cell responses can easily be monitored by measuring Ag-specific circulating Abs, T cell responses can only be gauged by biological tests such as cytotoxicity or proliferation. The absence of natural soluble TCR molecules in the blood and the low affinity of the TCR-MHC peptide interaction have been the main roadblocks to developing simple detection methods. The multimerization of MHC-peptide complexes answers partially this latter limitation by allowing an avidity effect and the staining of Ag-specific T cells (1, 2). However, this method, largely used for the detection of cytotoxic CD8+ T cells (3, 4), has limitations. The first one is essentially the geometry of display. Streptavidin is a rigid structure and the four biotin binding sites are positioned such that the MHC molecules are projected in the four cardinal directions (L. Teyton, unpublished observation) (5). These structural constraints prevent almost impossible the simultaneous engagement of the four MHC molecules (1). However, linear display of MHC molecules on synthetic polypeptides should lessen these steric problems (6). The second limitation of tetramers is still the low affinity or the low resulting avidity (2, 7). Indeed, the enhance-
Detachment of the peptide from the BSA enzyme according to the manufacturer’s instructions (Avidity, Denver, CO). Biota.

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Tetramer surface staining of T cell hybridomas was performed at various temperatures for 1–3 h with 10–100 μg/ml tetraro in FACS buffer (PBS plus 2% FCS plus 0.1% NaN₃). Liposome staining was done at room temperature for 1 h in FACS buffer. In all instances, tetratomer-coated liposomes were tested side by side. Single-cell suspensions prepared from OT-II-transgenic mice or OVA323–339-immunized mice were stained using the same protocol, followed by a 30-min staining at 4°C with anti-

Surface plasmon resonance

A BIAcore 2000 instrument (BIACORE, Piscataway, NJ) was used to determine interactions between purified MHC-peptide complexes and TCR molecules. Soluble OVA-2 TCR was immobilized on a CM5 research grade sensor chip by classic amine coupling. Injections of MHC/peptide molecules (I-Ab-OVA and I-Aβ-Exo) at the appropriate concentrations were performed in filtered and degassed PBS buffer at a flow rate of 20 μl/min. The data were analyzed using the BIAevaluation 3.0.1 software (BIACORE).

Confocal imaging on DO11.10 fixed cells

DO11.10 cells were incubated with I-Aβ-CLIP or I-Aβ-OVA tetrarmers-allophycocyanin coated on liposomes for 1 h at room temperature or 37°C to initiate activation prior to staining. After two washes in FACS buffer, cells were stained directly for 1 h on ice with a rabbit anti-allophycocyanin Ab (Biorad). Cells were then spun on glass slides using a cytospin expression system as previously described (20). Control peptides

Results

Soluble recombinant I-Aβ-OVA323–339 and I-Aβ-OVA323–339 molecules are functional but tetratomer do not stain specific hybridomas T cells

Soluble I-Aβ bearing a covalently attached peptide from OVA chicken (OVA323–339) and a leucine zipper were produced in a fly expression system as previously described (20). Control peptides were CLIP (23) and Exo (24). A BirA biotinylation sequence 85 (27) was added to the C terminus of the α-chain and molecules were biotinylated with the BirA enzyme after purification. Labeled I-Aβ-OVA323–339 tetratomers did not stain DO11.10 T cell hybridomas under any of the conditions tested: 4°C, room temperature, 37°C, 1–3 h, 10–100 μg/ml (Fig. 1A). Since, like in most cases within the OVA system, this cell line is cross-reactive on the B and

Preparation of liposomes

DPPC, cholesterol, DPPG, and DOGS-Ni-NTA or DPPE-X-Biotin, all in chloroform, were mixed at a molar ratio of 45:45:10:1. After evaporating the solvent under a stream of nitrogen gas, lipids were dried for 2 h in a Savant evaporator. The lipid film was rehydrated at 65°C in PBS at a concentration of 1 mg/ml. The suspension was extruded 11 times through 100-nm polycarbonate membranes using a mini-extruder (Avanti Polar Lipids). Stock liposome suspension was stored at 4°C. Liposomes (1 mg/ml, 30 μl per stain) were coated with recombinant MHC class II molecules (50 μg/ml for monomers, 20–50 μg/ml for trimers and tetramers) in 1.5 ml microcentrifuge tubes at room temperature for 1–2 h. Coating was done before each experiment.
**FIGURE 1.** Functional characterization of I-A<sup>b</sup>- and I-A<sup>d</sup>-OVA MHC molecules. A. Absence of tetramer staining of DO11.10 T cell hybridoma. The staining was performed for 3 h at 37°C with 50 µg/ml I-A<sup>b</sup> (left, solid line) or I-A<sup>d</sup>-OVA<sub>323-339</sub> (right, solid line) tetramers. Dotted curves correspond to the I-A<sup>d</sup>-CLIP control tetramer. B. The recombinant MHC class II molecules used to make tetramers are functional. DO11.10 or CLIP T cell hybridomas were cultured with various concentrations of plate-bound class II molecules (I-A<sup>b</sup>-CLIP (□), I-A<sup>d</sup>-CLIP (○)), or I-A<sup>d</sup>-OVA<sub>323-339</sub> (▲) for 24 h. Supernatant were harvested and tested for IL-2 presence using the IL-2-dependent NK cell line. Data are expressed as mean cpm of duplicate cultures.

- Absence of tetramer staining of DO11.10 T cell hybridoma. The staining was performed for 3 h at 37°C with 50 µg/ml I-A<sup>b</sup> (left, solid line) or I-A<sup>d</sup>-OVA<sub>323-339</sub> (right, solid line) tetramers. Dotted curves correspond to the I-A<sup>d</sup>-CLIP control tetramer. B. The recombinant MHC class II molecules used to make tetramers are functional. DO11.10 or CLIP T cell hybridomas were cultured with various concentrations of plate-bound class II molecules (I-A<sup>b</sup>-CLIP (□), I-A<sup>d</sup>-CLIP (○), or I-A<sup>d</sup>-OVA<sub>323-339</sub> (▲)) for 24 h. Supernatant were harvested and tested for IL-2 presence using the IL-2-dependent NK cell line. Data are expressed as mean cpm of duplicate cultures.

- **I-A<sup>b</sup>-OVA tetramers do not show high cooperativeness of binding as measured by surface plasmon resonance**

To try to understand the nonbinding of I-A<sup>b</sup>-OVA tetramers, we decided to measure the interaction between recombinant MHC and TCR molecules by using surface plasmon resonance (20). MHC monomers and tetramers were compared for their binding to the recombinant OT-II TCR (19) (Fig. 2A). The OT-II-specific TCR was produced in the same Drosophila expression system used for MHC production (20). The OT-II-specific TCR was engaged at any given time. Similar results were obtained with I-A<sup>d</sup>-OVA<sub>323-339</sub> molecules (data not shown). This absence of cooperation in binding was the most likely explanation to why functional MHC molecules could not bind T cells after tetramerization.

- **MHC class II-coated liposomes stain specifically T cell hybridomas**

To try to circumvent the low avidity of I-A<sup>b</sup>-OVA tetramers, we decided to display the same MHC molecules on a different platform. AAPC made with liposomes have been used successfully to stain and stimulate T cells (33). Lipid membranes offer molecular motion, large surface areas, larger number of MHC molecules and better geometry of molecular interactions than tetramers, and exclude other molecular species. Monomeric and tetrameric I-A<sup>a</sup>-EA molecules were used as negative control. The interaction between molecules was measured at a flow of 20 and 30 µl/min with similar results. The dissociation rate constant of monomers was measured at 4.68 µM with a relatively fast on rate (6.40 × 10<sup>3</sup> M<sup>-1</sup>s<sup>-1</sup>) and fast off rate (0.03 s<sup>-1</sup>; t<sub>1/2</sub>, 23 s), comparable to other TCR/MHC pairs (31–32) (Fig. 2A). I-A<sup>d</sup>-OVA<sub>323-339</sub> tetramers showed similar specific binding over the OT-II surface (Fig. 2B). Even though affinity numbers cannot be calculated for tetramers using common mathematical models, because of their variable valency, relative numbers, especially dissociation rates, can be approximated by global fitting. In the present case, the off rate was slowed down ~10 times (4.25 × 10<sup>3</sup> s<sup>-1</sup>; t<sub>1/2</sub>, 164 s) when compared with monomers (Fig. 2C). This limited increase was very suggestive that only two monomers within the tetramer could be engaged at any given time. Similar results were obtained with I-A<sup>d</sup>-OVA<sub>323-339</sub> molecules (data not shown). This absence of cooperation in binding was the most likely explanation to why functional MHC molecules could not bind T cells after tetramerization.
adaptable surfaces. Liposomes composed of DPPC, DPPG, cholesterol, DHPE-Bodipy, and DOGS-Ni-NTA or DHPE-biotin (molar ratio, 45:45:9:1) were used and decorated with MHC class II molecules. MHC molecules were coated to the liposomes surface using different approaches. Monomers were captured through their histidine tail using a chelating lipid (34). MHC trimers made by mixing at a 3:1 ratio MHC and SA-PE were attached using the fourth free SA site to a biotinylated lipid. Fluorescent MHC tetramers were captured via the histidine tail of the MHC. Liposomes were intrinsically labeled with Bodipy-DHPE or through the capture of fluorescent-labeled MHC multimers. As shown in Fig. 3A, I-A\(^{b}\)-OVA\(_{323-339}\) liposomes stained specifically DO11.10 hybridoma cells when compared with I-A\(^{b}\)-CLIP liposomes. However, the mean fluorescence intensity of the liposomes appeared lower than TCR expression, as tested with an anti-TCR/H9252-chain Ab (H57; Fig. 3A), but comparison could not be made since Bodipy vs PE was used as fluorophores, respectively. Liposomes coated with either MHC class II trimers-PE (Fig. 3B) or MHC class II tetramers-PE (Fig. 3C) gave a much higher mean fluorescence comparable to the level of TCR expression. These experiments indicated that the low affinity of monomers and the low avidity of tetramers of I-A\(^{b}\)-OVA\(_{323-339}\) could be rescued by displaying them at the surface of a liposome.

To quantify the number of liposomes bound per cell, DO11.10 hybridoma cells were stained with liposomes coated with MHC class II tetramers-allophycocyanin and examined by confocal microscopy. On average, 38 liposomes were bound per cell (data not shown) and we can calculate that each 100-nm liposome can display 60–200 MHC molecules. Therefore, a few hundreds to a few thousands MHC molecules can engage at the surface of one T cell. These numbers are in agreement with estimated numbers of TCR/MHC needed to trigger T cell activation (35).

Reversible staining of DO11.10 cells

A corollary to this approach is that the detachment of the MHC molecules from the liposome should lead to their detachment from the T cell and the loss of surface staining. This idea was tested directly by treating DO11.10 cells with imidazole buffer after liposome staining. Imidazole competes with histidine for the binding to Ni-NTA cages (36). DO11.10 cells were stained with I-A\(^{b}\)-OVA liposomes, washed, and incubated for 10 min with 200 mM imidazole solution (pH 7.5). After washing, cells were analyzed by flow cytometry. As seen in Fig. 4A, I-A\(^{b}\)-OVA\(_{323-339}\) liposomes staining of DO11.10 cells was completely abrogated after imidazole treatment. A similar experiment was conducted with one of the rare MHC class II tetramers that binds by itself with high functional affinity. The system used was the diabetogenic BDC2.5 T cell system for which we have developed I-A\(^{\gamma}\)-2.5 mimotopes that bind in their tetrameric form the specific T cell. In that instance, as expected, the disruption of the liposome-tetramer

interaction with imidazole did not alter staining significantly, although a slight shift of fluorescence toward the left was noticeable (mean fluorescence intensity before treatment, 35.7/mean fluorescent intensity after treatment, 23.7). These experiments demonstrated that the gain in avidity provided by liposomes is directly linked to the display of the tetrmeric by the liposome. In addition, the reversibility of staining provides the advantage of allowing MHC removal, a critical step to avoid activation if cells are to be used for additional functional studies (37).

Detection of naive T cells

Staining of T cells by MHC class II tetramers has been reported to require active metabolic processes and to be linked to T cell activation (8, 38–39). These observations linked the staining of MHC tetramers to the activation-induced redistribution of surface TCR molecules. We assumed that, independently of activation, liposomes should provide sufficient avidity effects to overcome scat-tering of TCR molecules on naive cells and to allow FACS staining. This idea was tested on unstimulated T cells from TCR-transgenic mice. The OT-II mice, transgenic for the OT-II TCR, constitutively express I-Ab–OVA 323–339 tetramers attached to liposomes stained 48.9% of the CD4+ T cells in the mouse presented in Fig. 5 compared with 48.5% of Vα2-positive CD4+ cells. Control stainings (SA alone and irrelevant tetramer) gave only minimal background. Therefore, naive T cells can be labeled with AAPC, independently of activation.

Detection of activated CD4+ T cells with AAPC

The following step was used to evaluate the efficiency of AAPC in identifying T cells in vivo after antigenic challenge. BALB/c and C57BL/6 mice were immunized with OVA 323–339 peptide in CFA and challenged 10 days later with the same peptide in IFA. Draining LN were harvested 4 days after the second immunization and single-cell suspensions were prepared and stained with I-Ab– and I-Aα–OVA 323–339 tetramer-coated liposomes and anti-CD4 Abs. Anti-CD8, anti-B2.20, and anti-CD11c Abs were used to exclude CD8+ T cells, B cells, and macrophages/dendritic cells. In BALB/c mice, 0.6% I-Ab–OVA 323–339–specific T cells were detected, whereas the I-Aβ–OVA 323–339 reagent detected 0.8% of positive cells (Fig. 6). On the C57BL/6 background, b vs d specificity appeared to be higher with 1.5% of I-Ab–OVA 323–339–specific CD4+ T cells and only 0.4% of I-Aα–OVA 323–339 cells. Background staining with an irrelevant liposome coated with I-Aβ–GP61 MHC molecules was ~0.2%, as was staining with tetramers alone. Similar nonspecific background was noted for non-CD4+ cells (Fig. 6, upper left quadrants) but varied greatly from experiment to experiment without exceeding 0.2%. To limit the extent of this
nonspecific staining, CD8⁺ T cells were always excluded by anti-CD8 Ab staining. These numbers show the paucity of natural OVA 323–339 T cells in BALB/c and C57BL/6 mice and their low avidity. The correlation between MHC class II liposome staining and intracellular cytokine staining was tested in the d haplotype by comparing percentages of liposome-positive cells and IFN-γ-producing CD4⁺ T cells (Fig. 7). Popliteal and inguinal BALB/c LN cells were restimulated in vitro with OVA 323–339 peptide or medium only. Exactly 0.4% of the CD4⁺ population expressed IFN-γ as tested by intracellular cytokine staining with anti-IFN-γ-allophycocyanin (Fig. 7, A and B). This percentage correlated well with the OVA-reactive T cell population detected with I-A^d-OVA 323–339 tetramers-liposomes (Fig. 7, C and D).

These results support strongly the idea that low-avidity CD4⁺ T cells are part of a normal immune response and would elude detection with tetramers.

Interaction of T cells with Ag-specific AAPC induces physiological capping at 37°C

To meet the criteria defining an AAPC, MHC-coated liposomes must be able to induce activation of interacting T cells at permissive temperatures. Activation of DO11.10 cells by liposomes was tested at 23 and 37°C by following formation of the immunological synapse using confocal microscopy. DO11.10 T cells were incubated with either I-A^d-OVA 323–339 or I-A^b-CLIP allophycocyanin-labeled tetramer-coated liposomes at either temperature, washed, and spun on slides by centrifugation. After fixation with 4% paraformaldehyde, cells were stained with either CD3-FITC or anti-Lck-FITC Ab after permeabilization with 0.1% saponin and observed by laser-scanning confocal microscopy. At 23°C, as shown in Fig. 8, B and D, CD3 and Lck, as well as I-A^b-OVA 323–339 liposomes, were evenly distributed around the cell surface and did not

FIGURE 6. AAPC detection of OVA-specific CD4⁺ T cells after OVA 323–339 peptide immunization. Cells were collected from popliteal and inguinal draining LN 4 days after the second immunization with peptide in BALB/c (top row) and C57BL/6 mice (bottom row). Staining is with I-A^b-OVA (middle panels), I-A^b-OVA (right panels), and I-A^b-GP 81 (left panels)-coated liposomes. Gating is on CD4⁺ T cells after exclusion of CD8-, B2.20-, and CD11c-positive cells. The percentage of positive cells in the gated population is indicated in the right corner of each histogram.
show signs of aggregation. To the contrary, at 37°C, cells became polarized with the capping of both CD3 and Lck together with the liposomes (Fig. 8, F and H). At the synapse, it is to be noted that cells formed a proboscis around which all of the bound liposomes collapsed without being internalized. In the presence of liposomes coated with I-A^b CLIP molecules, the irrelevant ligand, capping, and activation did not take place (Fig. 8, A and C, E and G). Similarly, fluorescent tetramers failed to bind and to initiate activation (data not shown).

In conclusion, liposomes can induce T cell activation and the formation of an immunological synapse at 37°C. They do not induce the redistribution of those same receptors at 23°C. This reconstitution of the physiological interactions between T cells and APC qualifies the liposomes as true AAPC. They can be used as staining reagents at low temperatures and surrogate APC at 37°C.

**Discussion**

The detection/isolation of Ag-specific CD4^+ T cells is still a practical challenge of functional immunology (1, 2). Following and monitoring spontaneous and postvaccinal immune responses remain elusive and limited to a handful of systems where TCR-transgenic mice are available. This paradigm has marginally benefited from the development of MHC tetramers that has been so successful for the detection of Ag-specific CD8^+ cytotoxic T cells (3, 4). The reasons for the discrepancy between MHC class I and MHC class II tetramers are still unclear. It is probably the convergence of many parameters: 1) low frequency of CD4^+ T cells (11, 12, 16, 17), 2) low affinity of MHC-TCR interactions, 3) difficulties in the preparation of soluble class II-peptide complexes, 4) lower affinity of MHC class II-binding peptides as compared with class I-binding peptides, 5) sliding of the peptide within the groove (28, 40, 41), 6) importance of the flanking residues for binding and recognition, 7) nonequivalent functions of CD4 and CD8 accessory molecules, and 8) need to have active metabolic processes for staining (38). More importantly, it points out the fundamental differences between CD4^+ and CD8^+ T cells in term of physiology.

**FIGURE 8.** AAPC induce Ag-specific activation of T cells at 37°C. DO11.10 hybridoma T cells were incubated at 25°C (A–D) or 37°C (E–H) with I-A^b-CLIP or I-A^b-OVA_{323-339} AAPC for 1 h and washed in PBS. For analysis, cells were spun on slides and fixed with 4% paraformaldehyde. Staining was done with anti-CD3 (C, D, G, and H) or anti-Lck Abs (A, B, E, and F). Colors were electronically switched to green for liposomes and red for Ab.
This article demonstrates once again the limit of the tetramer technology and proposes an alternative system for detecting MHC class II tetramer-negative CD4⁺ T cells. However, the goal is not to oppose two techniques but to build complementary tools that allow detection of CD4⁺ T cells. In the experimental system that we have chosen in the present report, the OVA system, we were able to demonstrate that the tetramers were not working because the multivalency of the molecule did not translate into a sufficient gain of affinity. It is likely that only two of the four sites engage at any given time to result in the 10 times gain in half-life that was observed. The apparent non- engagement of the other two sites could be explained by the geometry of the TCR-MHC interaction. In the case of MHC class I-TCR interactions, the accumulation of structural data within different systems has clearly established that a diagonal orientation of TCR over MHC was the rule with only little variation between systems (31, 32). MHC class II-TCR interactions have not been so well documented, with only three structures determined, but it is already clear that TCR orients over MHC in a more orthogonal footprint (42). The transition from diagonal to orthogonal axis could limit the simultaneous engagement of more than two sites for geometrical constraints within the SA-MHC complex. Geometrical constraints have also been the basis to explain the variable cooperativeness of binding between the two Fab sites of Igs (43–46). In that instance, cooperation leads to functional affinities that can vary from 10– to 100-fold between Fab and IgG (44, 46). Alternatively, the limitation could come from the fast off rate (0.03 s) which could impede the simultaneous engagement of three sites at any given time, with the dissociation of either of the first two sites whenever the third site becomes engaged and translate in the absence of cross-linking. In that respect, the 0.03 s could be considered as the threshold to observe or not cooperativeness of binding. In vivo, these two parameters will combine to create the diffusion/cross-linking coefficient (or \( K_C \)) of TCR molecules within the plasma membrane (47). Liposomal display of MHC molecules bypasses some of these limitations of tetramers through three distinct mechanisms. First, individual liposomes will display 60–200 MHC molecules instead of only 4, each of which can individually engage in a productive interaction and create the resulting “avidity effect.” Second, the freedom of motion of MHC molecules at the surface of liposomes will limit any geometrical limitations that the tetramers may endure. Third, these MHC molecules will have lateral mobility within the membrane layer and facilitate reciprocal cross-linking of MHC and TCR molecules on the two opposite membrane surfaces. We established that membrane display and mobility of MHC molecules were critical parameters to get proper TCR binding by showing that MHC molecules displayed at the surface of polystyrene beads (48) did not, despite their numbers, bind to CD4⁺ T cells (data not shown). The usage of liposomes as potential AAPC was reported recently with B cell-purified MHC molecules (33). However, the natural source of MHC molecules and the difficulties of loading peptide onto them were great limitations of the approach that only recombiant technology could bypass. The most efficient display of the MHC class II molecules that we tested in our system was to capture SA/MHC trimers by using a biotinylated lipid and the fourth binding site of the SA molecule. However, the inconsistencies between batches and between manufacturers in respect to the molar ratio of SA/PE and valency of coupled SA make this approach impractical. Monomer-coated liposomes had the limitations of using Bodipy fluorophores instead of PE, therefore limiting their brightness. Efforts to produce PE liposomes are under way.

In a practical situation such as the OVA system that we examined, we were able to show that the liposomes were specific and could bind to hybridoma T cells as well as naive T cells from transgenic animals and postimmunization T cells. As expected for CD4⁺ T cells, frequencies were low after immunization (0.8–1.5%) and tested the limit of detection of the technique. Background levels of 0.1–0.2% are still current in our hands but could eventually be improved by using better negative controls than “irrelevant” immunogenic peptides. Indeed, if the method is sensitive enough it should be able to detect cognate T cells of low frequency and the questions become “What is background, what is specific?” To answer these questions, peptides with no up-facing residues are being designed and will be tested to determine “real” background. Beyond the detection of CD4⁺ T cells that cannot be detected by tetramers, we clearly demonstrated that liposomes displaying MHC molecules could be used as tools for T cell activation studies. The formation of a molecular cap with recruitment of CD3 and Lck without internalization of the liposomes reconstitute the formation of the immunological synapse. It gives the unique opportunity of studying the polarization of the T cell during activation. The addition of accessory molecules such as B7 and ICAM to the surface of the liposome will enable us to study similar phenomena for naïve T cells. It also brings the exciting possibility of using the liposomes as in vivo AAPC for activation or removal of specific T cells.

In conclusion, we have used AAPC built with liposomes to detect CD4⁺ T cells that could not be isolated using MHC class II tetramers. AAPC lead to T cell activation at 37°C and to the full reorganization of the TCR complex and polarization of the T cell. AAPC will be important tools for ex vivo detection of CD4⁺ T cells and in vitro functional and molecular activation studies.

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