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Technical note

A stepwise protocol to coat aAPC beads prevents out-competition of anti-CD3 mAb and consequent experimental artefacts

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ABSTRACT

Artificial antigen-presenting cells (aAPC) are widely used for both clinical and basic research applications, as cell-based or bead-based scaffolds, combining immune synapse components of interest. Adequate and controlled preparation of aAPCs is crucial for subsequent immunoassays. We reveal that certain proteins such as activatory anti-CD3 antibody can be out-competed by other proteins (e.g. inhibitory receptor ligands such as PDL1:Fc) during the coating of aAPC beads, under the usually performed coating procedures. This may be misleading, as we found that decreased CD8 T cell activity was not due to inhibitory receptor triggering but rather because of unexpectedly low anti-CD3 antibody density on the beads upon co-incubation with inhibitory receptor ligands. We propose an optimized protocol, and emphasize the need to quality-control the coating of proteins on aAPC beads prior to their use in immunoassays.

1. Introduction

Artificial antigen-presenting cells (aAPC) are frequently used in immunoassays for several purposes, primarily due to their capacity to efficiently induce T cell activation under controlled stimulatory conditions. Two major classes of aAPC exist, either cell-based or bead-based aAPC (Kim et al., 2004; Turtle and Riddell, 2010), that act as scaffolds onto which particular immune synapse components are added. One example of cell-based aAPC is the K562 cell line, which lacks major elements of the immune synapse, and can be modified to express particular MHC molecules and co-stimulatory proteins, or decorated with activatory anti-bodies and Fc chimeras of ligands via CD32-mediated binding. The second class of aAPC are based on cell-sized particles such as vesicles or beads (Kim et al., 2004; Turtle and Riddell, 2010) that can be manipulated to display for instance T cell activatory anti-CD3 monoclonal antibodies (anti-CD3 mAb) or peptide/MHC complexes, together with co-stimulatory molecules, by chemical immobilization on beads or by aggregation in vesicle membranes.

Due to the possibility to easily manipulate and control T cell-stimulatory conditions and their versatility, aAPCs are used in numerous applications, including protocols for the preparation of T cells for adoptive transfer immunotherapy (e.g. the activation and expansion of T cells ex vivo) (Turtle and Riddell, 2010). In addition to the clinical applications, aAPC offer a technically advantageous system to study basic mechanisms of T cell activation and function, for instance by coating aAPC beads with immune synapse components of interest, in particular and controlled combinations and doses (Blair et al., 1998; Broeren et al., 2000; Carter et al., 2002; Chemnitz et al., 2004; Sheppard et al., 2004; Parry et al., 2005; Butte et al., 2007; Francisco et al., 2009).

In this study, we used aAPC beads coated with multiple proteins, using a single protein mix, according to the usually performed standard procedures. Unexpectedly, we observed that certain proteins may out-compete other proteins when incubated simultaneously. We optimized the coating of the beads and illustrate that, unless the efficiency of protein coating is checked prior to the use of aAPC in immunoassays, such out-competition may lead to erroneous conclusions.

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2. Methods

2.1. aAPC beads

The beads used were Dynabeads® M-450 Epoxy (Invitrogen™). These are cell-sized (4.5 μm), paramagnetic, hydrophobic and covered with epoxy groups, which irreversibly react with amine, thiol and hydroxyl groups to immobilize proteins on the bead surface. The coating of beads was performed by incubation in 0.1 M PO_4 (pH 7.5) for 1 h at RT, with agitation, at a density of 2×10^7 beads per ml. The manufacturer recommends to incubate for 16–24 h, but we found that 1 h is sufficient and leads to similar capacity of the anti-CD3-coated beads to stimulate T cells (this is particularly useful for the stepwise procedure described below). Coating was followed by three washes in PBS and by resuspension in complete medium (composition described in "CD8 T cell stimulation") overnight at 4 °C. As a standard, 10^7 beads (500ul) were coated with anti-CD3 monoclonal antibody (hybridoma clone OKT3) at an effective concentration of 75% (EC75, based on preliminary titration experiments) using 0.3 μg (8.3 nM) of OKT3, and with 50 nM of iRLs : 1.3 μg of PDL1:Fc (RnD Systems®), and 2.43 μg of HLA-DR1 (in house). The Fc portion of human IgG1 (BioXCell) was used as a control and as a «filler» to cover beads with a constant amount of proteins, completing the mixes to 5 μg of total protein per 10^7 beads. These conditions fall in the range of parameters described previously in the literature (Blair et al., 1998; Broeren et al., 2000; Carter et al., 2002; Chemnitz et al., 2004; Sheppard et al., 2004; Parry et al., 2005; Butte et al., 2007; Francisco et al., 2009) and in the manufacturer's protocol. When the modified, stepwise coating procedure was used, the total 5 μg of protein was distributed between the different coating steps (e.g. 2.5 μg per step using a two-step protocol), with 1 h of incubation per step. Beads were washed once in PBS between steps, with three final washes in PBS and resuspension in complete medium as above.

2.2. CD8 T cell stimulation

Complete medium was RPMI 1640, complemented with 10% heat-inactivated FCS, 1% non-essential aminoacids (Gibco), 1% L-glutamine (Gibco), Hepes (10 mM) and 10,000 U/ml of penicillin/streptomycin (Gibco). Human CD8 T cell clones (clone 618–5 and clone 1013–3.11) were generated from circulating blood of melanoma patients LAU 618 and LAU 1013, cultured in 150 U per ml of IL-2 (Speiser et al., 2011). Peripheral blood mononuclear cells (PBMC) were obtained from healthy donor volunteers following density gradient fractionation using Lymphoprep™. CD8 T cell clones or PBMC were stimulated with beads at a 2:1 ratio (beads:T) and a density of 10^6 cells per ml in complete medium, using flat-bottom culture plates. For the proliferation assays, 0.5 μg/ml of PHA was used as a control.

2.3. Flow cytometry

To quantify the proteins coated on beads, antibodies used were sheep anti-mouse FITC (Chemicon) to detect OKT3, anti-human PDL1 PE-Cy7 (clone MIH1, BD Biosciences) to detect PDL1:Fc, anti-human HVEM PE (clone 122, Biolegend) to detect HVEM:Fc, and anti-HLA class II FITC (clone Bu26, Abcam) to detect HLA-DR1. For each protein, the % efficiency of coating was calculated on the basis of the geometric mean of fluorescence intensity (GeoMFI) standardized using beads coated with the respective protein (=100%) and uncoated or Fc-coated beads (=0%, as indicated). Note that the strength of the detection signal depends on both the quantity of protein coated and the antibody used for detection, and only a relative quantification of coating, comparing different protein mixes used, is obtained (i.e. this method does not yield an absolute quantitation of protein coating). T cell activation was analyzed as follows: 1) anti-human CD107a FITC (clone H4A3, BD Biosciences) was added during the co-culture of T cells with aAPC beads; 2) cells were surface stained using anti-human CD8 Pacific-Blue (clone B9.11, Beckman Coulter); 3) dead cell exclusion was done using the fixable dead-cell marker Vivid-Aqua (Molecular Probes®, Invitrogen); 4) following fixation of cells in 1% formaldehyde, intracellular cytokines were labeled using anti-human TNFa Alexa-700 (clone Mab11, BD Biosciences) and anti-human IFNg PE-Cy7 (clone 4SB3, BD Biosciences) in FACS buffer (PBS with 5 mM EDTA and 0.2% BSA), supplemented with 0.1% saponin. For proliferation measured by CFSE dilution, cells were stained at 5×10^6 cells/ml in 0.25uM CFSE (Molecular Probes®, Invitrogen) in PBS for 7 min at 37 °C followed by addition of 1× volume of cold FCS and incubation on ice for 5 min, one wash in complete medium, incubation in medium on ice for 15 min to equilibrate the CFSE, and one last wash prior to the final resuspension in complete medium for T cell stimulation. Data acquisition was performed with Gallios flow cytometer (Beckman Coulter) and the data was processed with FlowJo (Tree Star Inc.) software.

3. Results

Our initial goal was to setup a system where controlled doses and combinations of co-inhibitory receptor ligands (iRLs) can be used in the context of anti-CD3 stimulation of human CD8 T cells, in order to study the immunodulatory capacity of various iRLs and their potentially synergistic action. We therefore used recombinant iRLs such as PDL1:Fc (PD1 ligand) and soluble HLA-DR1 (LAG-3 ligand) to coat aAPC beads, together with activatory anti-CD3 mAb (Fig. 1A), using different mixes of the various proteins, according to a standard coating procedure (Methods section) recommended by the manufacturer of the beads. aAPC beads coated with anti-CD3 mAb alone efficiently induced T cell activation (Fig. 1B and C), as the T cells readily produced IFNg and TNFa, and translocated CD107a to the surface (degranulation). Strikingly, the extent of activation was dramatically lower in T cells stimulated with aAPC beads coated with anti-CD3 mAb together with PDL1:Fc or HLA-DR1, affecting both cytokine secretion and degranulation (Fig. 1B and C). At first sight, this result corresponded to the hypothesis that iR triggering may decrease T cell activity, in agreement with previous reports particularly on the effect of the PD1:PDL1 axis on T cell function using aAPC beads (Carter et al., 2002; Chemnitz et al., 2004; Sheppard et al., 2004; Parry et al., 2005; Butte et al., 2007; Francisco et al., 2009). However, a more detailed characterization of the aAPC beads by flow cytometry revealed that the presence of iRLs strongly impaired