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J Immunol Methods. Author manuscript; available in PMC 2013 October 31.

Published in final edited form as:
J Immunol Methods. 2012 October 31; 384(1-2): 184–190. doi:10.1016/j.jim.2012.07.011.

PE-Cy5.5 conjugates bind to the cells expressing mouse DEC205/CD205

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Abstract

DEC205/CD205, an endocytic receptor of C-type multilectin, is expressed highly in dendritic cells (DCs). DEC205 was shown to efficiently deliver vaccine antigens in surrogate ligands to the antigen processing and presentation machinery of DCs, which resulted in the development of DC-targeted vaccines employing anti-DC monoclonal antibodies (mAbs). During our studies to characterize a variety of anti-DC mAbs including anti-DEC205 by flow cytometric analysis, we discovered that a secondary anti-immunoglobulin antibody conjugated with PE-Cy5.5 bound strongly to the cells expressing mouse DEC205 (mDEC205) without incubation of a primary anti-mDEC205 mAb. In the present study we demonstrate that various antibodies and streptavidin conjugated with PE-Cy5.5 bind to the mDEC205-expressing cells including CHO, KIT6, and HEK293 cells. The interaction between the PE-Cy5.5 conjugates and the cells expressing mDEC205 appears distinctive, since none of PE-Cy5.5 conjugates bind to the cells that express human DEC205 on surface. Besides, only PE-Cy5.5 conjugates bind strongly to mDEC205-expressing cells; PerCP-Cy5.5, APC-Cy5.5, and Cy5.5 conjugates bind weakly; PE, PE-Cy5, Cy5, FITC, or Alexa488 conjugates do not bind. Therefore the use of PE-Cy5.5 conjugates, widely utilized in multicolor flow cytometry, requires precaution against nonspecific binding to mDEC205-positive cells.

Keywords

CD205; DEC205; PE-Cy5.5; Nonspecific binding; Dendritic Cells

1. Introduction

Dendritic cells (DCs) are localized in the T-cell areas of lymphoid tissues, where DCs are essential to orchestrate the immune system to tolerate or respond appropriately to an enormous number of diverse challenges. Many potential endocytic receptors are identified on DC surface, which not only play important roles in efficient uptake, processing, and presentation of antigens but also mark the distinct subsets of DCs (Steinman, 2012). To harness DCs to boost or regulate immune system, a number of antigens have been targeted

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to several DC-specific endocytic receptors by exploiting monoclonal antibodies (mAbs) beginning with anti-DEC205/CD205 (Hawiger et al., 2001; Steinman, 2012).

DEC205 is a C-type multilectin receptor which is abundantly expressed in DCs. DEC205 was shown to carry out endocytosis of antigens within anti-DEC205 mAb efficiently and thus mediate proficient processing and presentation of antigens in DCs (Trumpfheller et al., 2012). This approach of targeting vaccines selectively to DCs by integrating antigens into anti-DEC205 mAb has been extended to other anti-DC mAbs, such as anti-DCIR2, anti-Langerin/CD207, and anti-CLEC9A/DNGR1 (Idoyaga et al., 2011). During our studies to characterize a variety of anti-DC mAbs including anti-DEC205, anti-Langerin, and anti-DC-SIGN/CD209 by flow cytometric analysis (Cheong et al., 2007; Cheong et al., 2010a; Park et al., 2012), we unexpectedly discovered that a secondary anti-immunoglobulin antibody conjugated with PE-Cy5.5 bound strongly to the cells expressing mouse DEC205 (mDEC205) without any prior treatment of a primary anti-mDEC205 mAb, which we now describe in this report. A similar phenomenon was reported that Cy5-based conjugates, including antibodies containing Cy5 and PE-Cy5, bound to the cells transfected with human CD64 (hCD64), the high affinity receptor for IgG, but not to the cells untransfected or transfected with other human Fc receptors (Van Vugt et al., 1996; Jahrsdörfer et al., 2005).

In this study we demonstrate that antibodies and streptavidin conjugated with PE-Cy5.5 bind nonspecifically, i.e. irrespective of their specificity, to the cells expressing mDEC205 but not to those expressing human DEC205 (hDEC205) or else. The strong binding between mDEC205-expressing cells and PE-Cy5.5 conjugates appears distinctive, since other Cy5.5-based conjugates bind only weakly to mDEC205-positive cells.

2. Materials and methods

2.1. Cells and expression of lectins

Chinese hamster ovary (CHO) cells (CHO-S cells, Invitrogen, Carlsbad, CA), KIT6 mouse T-cell hybridomas (Park et al., 1996), human embryonic kidney 293 (HEK293) cells, and hybridomas for NLDC145 (anti-mDEC205), MG38 (anti-hDEC205), and 33D1 (anti-mDCIR2) mAbs were cultured in DMEM (Gibco Invitrogen, catalog number 11995) with 7 % fetal bovine serum (FBS) or 5% Ultra-Low IgG FBS (Invitrogen) supplemented with 1× solutions of non-essential amino acids and antibiotic-antimycotic (Invitrogen).

The generation of stable CHO cell lines expressing full-length hDEC205 (CHO/hDEC205) and mDEC205 (CHO/mDEC205) has been described previously (Cheong et al., 2010b). Stable CHO cell lines expressing mouse DCIR2 (CHO/mDCIR2) were generated similarly to CHO/hDEC205 and CHO/mDEC205 cells. In brief, a cDNA encoding full-length DCIR2 was cloned from C57BL/6 mouse spleen, inserted into pCMV expression vector (Clontech, Mountain View, CA), transfected to CHO cells, and stably expressed as CHO/mDCIR2 cells. KIT6 T-cell hybridoma lines stably expressing full-length mDEC205 (KIT6/mDEC205) or mouse MHC class I Kb alpha chain (KIT6/Kb) were generated by retroviral transduction using a pMX vector (Onishi et al., 1996) and a full-length cDNA of mDEC205 or Kb, following the procedure described previously (Park et al., 1996). HEK293 cells were transiently transfected with the plasmid DNA of pCMV expression vector encoding mDEC205, hDEC205, mDCIR2, or no insert by using Lipofectamine™ 2000 reagent (Invitrogen) at 24 hrs prior to the flow cytometric analyses as below.

2.2. Reagents

We purchased Cy5.5-conjugated and Cy5-conjugated anti-rat IgG (goat polyclonal) from Novus Biologicals (Littleton, CO); PE-conjugated anti-rat IgG (goat polyclonal), PE-conjugated anti-mouse IgG1 (goat polyclonal), PE-conjugated anti-rabbit IgG (goat

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polyclonal F(ab′)2), and PE-Cy5.5-conjugated anti-rat IgG (goat polyclonal F(ab′)2) from Southern Biotech (Birmingham, AL); PE-Cy5.5-conjugated streptavidin, PE-Cy5.5-conjugated anti-mouse CCR7 (rat IgG2a/kappa), PE-Cy5-conjugated anti-mouse CD80 (Armenian hamster IgG), PerCP-Cy5.5-conjugated anti-mouse interferon-gamma (rat IgG1/kappa), and PerCP-Cy5.5-conjugated anti-B220 (rat IgG2a/kappa) from eBioscience (San Diego, CA); PE-Cy5.5-conjugated, PerCP-Cy5.5-conjugated, APC-Cy5.5-conjugated, and PE-Cy5-conjugated mouse IgG2a/kappa isotype control from BioLegend (San Diego, CA); PerCP-Cy5.5-conjugated anti-mouse CD25 (rat IgG1/lambda), and PerCP-Cy5.5-conjugated anti-mouse CD4 (rat IgG2a/kappa) from BD Biosciences (San Jose, CA). NLDC145, MG38, and 33D1 mAbs were purified from culture supernatants of individual hybridomas. Purified mAbs were conjugated with fluorochrome using Alexa488 (Invitrogen) or FITC (Pierce Biotechnology, Rockford, IL) labeling kits following the manufacturer's instruction.

2.3. Flow cytometry

Adherent (CHO and HEK293) cell lines were detached using 1 mM EDTA in PBS. KIT6 or detached cells were washed once with PBS containing 2 % FBS (FACS buffer) and then incubated with respective fluorochrome conjugates at the concentration of 0.05 – 5 μg/ml in FACS buffer at 4 °C for 30 min, followed by wash with FACS buffer. Fluorescence of the cells was measured by BD FACSCalibur or BD LSR II flow cytometer and analyzed with FlowJo (Tree Star, Ashland, OR) at the Rockefeller University Flow Cytometer Resource Center.

3. Results

3.1. Nonspecific binding of PE-Cy5.5 conjugates to the CHO cells expressing mDEC205

We generated and characterized a variety of anti-DC mAbs, especially against a series of DC-specific C-type lectin receptors, including anti-DEC205, anti-Langerin, and anti-DC-SIGN (Cheong et al., 2007; Cheong et al., 2010a; Park et al., 2012). During these studies, we purchased and tested a goat polyclonal anti-rat IgG conjugated with PE-Cy5.5 (from Southern Biotech) for the flow cytometric detection of anti-DC mAbs. Unexpectedly, we discovered that this secondary anti-rat IgG/PE-Cy5.5 conjugate bound strongly to the CHO cells expressing mDEC205 (CHO/mDEC205) with or without prior incubation of a primary anti-mDEC205 mAb, NLDC145 (rat IgG2a/kappa), but not to other cells (Fig. 1A), where all incubations were performed at 4 °C for 30 min. To investigate whether the nonspecific binding of this PE-Cy5.5-conjugated antibody to CHO/mDEC205 cells is due to PE-Cy5.5 conjugation, 3 more PE-Cy5.5 conjugates from different commercial sources (anti-mouse CCR7 and streptavidin from eBioscience; mouse IgG2a/kappa isotype control from BioLegend) were tested. As shown in Fig. 1B, all PE-Cy5.5-conjugated antibodies and streptavidin bound strongly to CHO/mDEC205 cells but not to control cells. Since all 4 PE-Cy5.5 conjugates used in the experiments were distinct (i.e., a goat polyclonal, a rat monoclonal, a mouse monoclonal, and a streptavidin) and from 3 different commercial suppliers (i.e., Southern Biotech, eBioscience, and BioLegend), we concluded that PE-Cy5.5 caused the conjugates to bind CHO/mDEC205 cells irrespective of the specificity of antibodies and streptavidin.

3.2. Binding of PE-Cy5.5 conjugates to cells in proportion to mDEC205 expression on surface

Now that all PE-Cy5.5 conjugates bound clearly to CHO/mDEC205 cells, we examined whether mDEC205-posive cell lines other than CHO cells could also bind to PE-Cy5.5 conjugates. We generated transfectant KIT6/mDEC205 cells, mouse T-cell hybridoma cells that stably express mDEC205 on surface. We also had HEK (human embryonic kidney) 293 cells transfected transiently with the mammalian expression vector encoding a full-length

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cDNA of mDEC205. Both KIT6 and HEK293 cells transfected with mDEC205 were able to bind to PE-Cy5.5 conjugates similarly as CHO/mDEC205 cells, whereas the control transfectant cells were not (Figs. 2A and 2B). Therefore, it is evident that mDEC205 expressed on the surface of mouse KIT6, human HEK293, and hamster CHO cells is responsible for those cells to bind PE-Cy5.5 conjugates.

Then we investigated if the expression level of mDEC205 on cell surface correlated to the capacity of cells to bind PE-Cy5.5 conjugates. We selected CHO/mDEC205 cell lines with broad expression levels of mDEC205 on surface. These CHO/mDEC205 cells were incubated with both PE-Cy5.5 conjugate and Alexa488-conjugated anti-mDEC205 NLDC145. As shown in Fig. 2C, more PE-Cy5.5 conjugate bound to those cells that expressed higher levels of mDEC205 on surface (also see Fig. 3B). Hence, the binding of PE-Cy5.5 conjugate to cells is directly proportional to the level of mDEC205 expression on cell surface.

3.3. PE-Cy5.5 conjugates do not bind nonspecifically to hDEC205-positive cells

The sequences of DEC205 from various species, including both mammals and non-mammals, exhibit a lot of highly conserved regions of identical amino acids in the extracellular domain (Guo et al., 2000; Gliddon et al., 2004; Nagasawa et al., 2010). Therefore, it is of interest to examine whether PE-Cy5.5 conjugates bind to the cells expressing DEC205 from species other than mouse. We had cloned and expressed human DEC205 (hDEC205) in cell lines (Fig. 3A) previously (Cheong et al., 2010b; Park et al., 2012), so we could compare the binding of PE-Cy5.5 conjugates between mDEC205-positive and hDEC205-positive cells. An equal number of CHO/mDEC205 and CHO/hDEC205 cells were mixed together and incubated with PE-Cy5.5 conjugate plus anti-hDEC205 or anti-mDEC205 mAb. Clearly, PE-Cy5.5 conjugate was not able to bind CHO/hDEC205 cells, whereas PE-Cy5.5 conjugate bound CHO/mDEC205 cells in direct proportion to the level of mDEC205 expression on surface (Fig. 3B). In addition, PE-Cy5.5 conjugates were not able to bind HEK293 cells transfected with hDEC205 (data not shown).

3.4. Weak nonspecific binding of PerCP-Cy5.5, APC-Cy5.5, and Cy5.5 conjugates to mDEC205-positive cells

In the experiments, thus far, we used conjugates containing PE, FITC, or Alexa488, beside PE-Cy5.5, and we found that only PE-Cy5.5 conjugates bound nonspecifically to mDEC205-positive cells. Since it would be important to identify any other fluorochrome conjugates that bound to mDEC205-positive cells, we tested a series of different fluorochrome-conjugated antibodies for nonspecific binding. As shown in Fig. 3C, mDEC205-positive cells bound nonspecifically to antibodies conjugated with PerCP-Cy5.5 and APC-Cy5.5 to a lesser extent than those conjugated with PE-Cy5.5. We also compared the antibodies conjugated with Cy5.5 or Cy5 for their nonspecific binding to mDEC205-positive cells, and found that only Cy5.5-conjugated antibody bound mDEC205-positive cells to a lesser extent than PE-Cy5.5 conjugate (Fig. 3C). In the meantime, none of the conjugates that we have tested so far were able to bind nonspecifically to hDEC205-positive cells (Fig. 3C).

4. Discussion

During the experiments to generate and characterize a variety of anti-DC mAbs, we unexpectedly discovered that PE-Cy5.5 conjugates bound strongly to the cells expressing mDEC205 on surface. Then, we determined that various antibodies and streptavidin conjugated with PE-Cy5.5 bound nonspecifically to the cells in direct correlation with the expression level of mDEC205. Previously, others reported similar findings that antibodies

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conjugated with Cy5 or PE-Cy5 bound nonspecifically to hCD64-positive cells (Van Vugt et al., 1996; Jahrsdörfer et al., 2005). Unlike the interaction between hCD64-positive cells and Cy5-based conjugates, we find that neither Cy5.5 nor PerCP-Cy5.5 nor APC-Cy5.5 conjugates bind strongly to mDEC205-positive cells as PE-Cy5.5 conjugates. This implies that the ligand structure required for PE-Cy5.5 conjugates to bind mDEC205 receptor resides not only in Cy5.5 but also in PE molecules. Thus far, no natural ligand structures for mDEC205 receptor have been characterized although some microbial and endogenous molecules were suggested to bind mDEC205 (Sancho and Reis e Sousa, 2012). Since certain forms of deoxynucleotides were shown to block the interaction between hCD64 and Cy5-based dyes (Jahrsdörfer et al., 2005), it might be interesting to explore if any deoxynucleotides can bind to mDEC205 as well. Further researches on the direct binding between mDEC205 and PE-Cy5.5 will likely reveal the information on how mDEC205 interacts with its ligand.

We carried out the analyses to show strong binding between PE-Cy5.5 conjugates and mDEC205 in transfectant cell lines, but not in endogenous cells, i.e. DEC205-positive mouse DCs. Given that DCs are abundant in a variety of pattern recognition receptors, the difference in binding to PE-Cy5.5 conjugates between mDEC205-positive and mDEC205-negative DCs might not be as conspicuous as that between mDEC205-positive and mDEC205-negative transfectant cell lines. It also needs to be noted that the levels of mDEC205 expression on transfectant cell lines are higher than those found on endogenous DCs. In conclusion, the use of PE-Cy5.5 conjugates, such as antibodies and streptavidin, requires precaution against nonspecific binding to mDEC205-positive cells in flow cytometry.

Acknowledgments

We thank all members of Steinman Laboratory for their excellent support and valuable discussion. We were supported by NIH Grants to RMS (AI13013, AI40045) and CGP (AI093216).

Abbreviations

Ab	antibody
CHO cells	Chinese hamster ovary cells
DCs	dendritic cells
FBS	fetal bovine serum
hCD64	human CD64
hDEC205	human DEC205
HEK293 cells	human embryonic kidney 293 cells
mAb	monoclonal antibody
mDCIR2	mouse DCIR2
mDEC205	mouse DEC205

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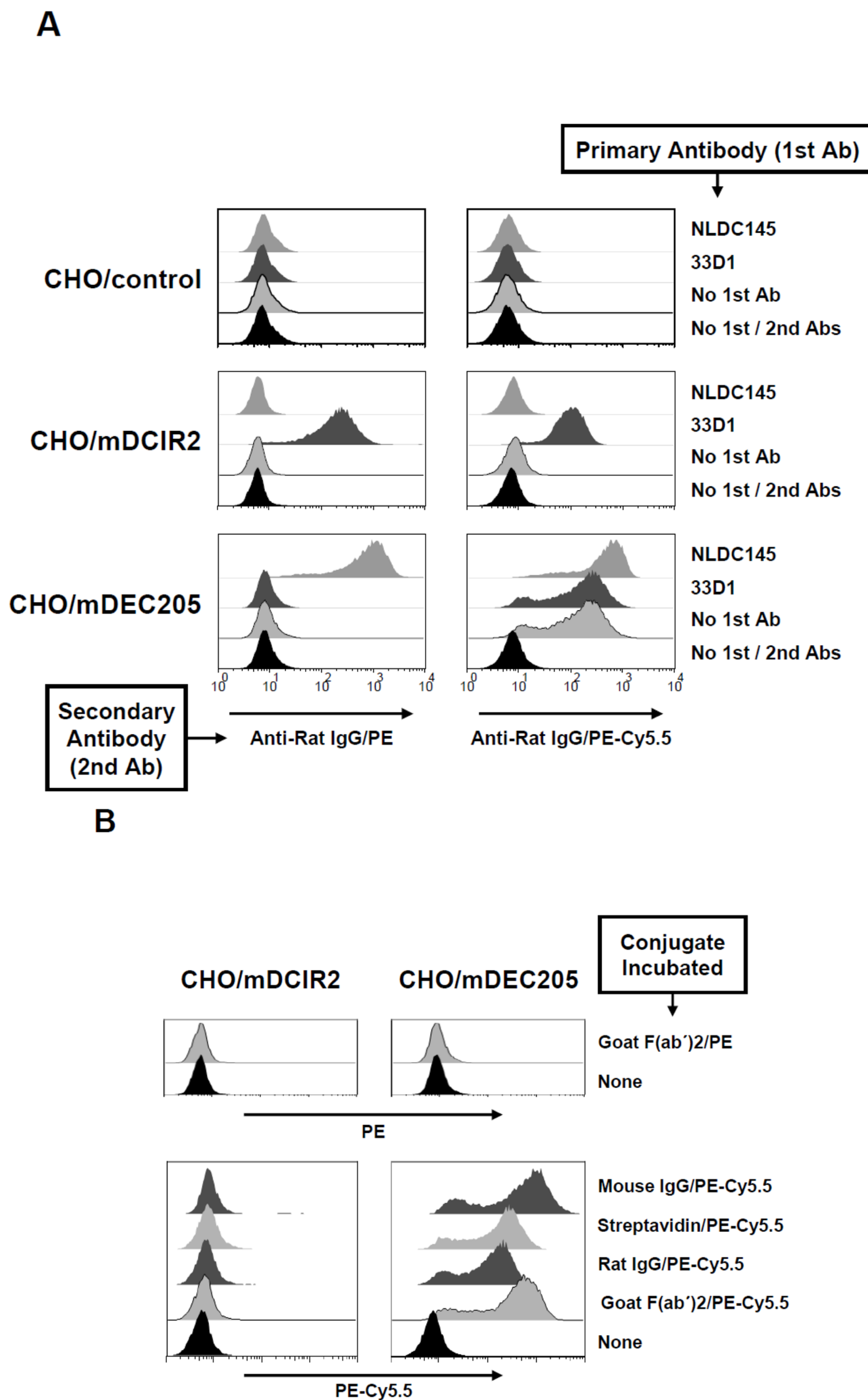

Fig 1. Nonspecific binding of PE-Cy5.5 conjugates to mouse DEC205-positive CHO cells

(A) CHO cells untransfected (CHO/control) or stably transfected with mouse DCIR2 (CHO/mDCIR2) or mouse DEC205 (CHO/mDEC205) were incubated with/without the primary

antibody (1st Ab) supernatant of anti-mDEC205 (NLDC145) or anti-mDCIR2 (33D1). Then, the cells were washed and incubated with/without each fluorochrome-conjugated secondary antibody (2nd Ab) at 0.25 μg/ml, followed by flow cytometry. Every incubation procedure was performed at 4 °C for 30 min throughout this study.

(B) CHO/mDCIR2 and CHO/mDEC205 cells were incubated with/without each fluorochrome-labeled conjugate, antibody or streptavidin, at 0.25 μg/ml followed by flow cytometry. Antibodies are noted for their species origin and isotype (see materials and methods for detail), and their specificity is relevant to neither mDEC205 nor mDCIR2.

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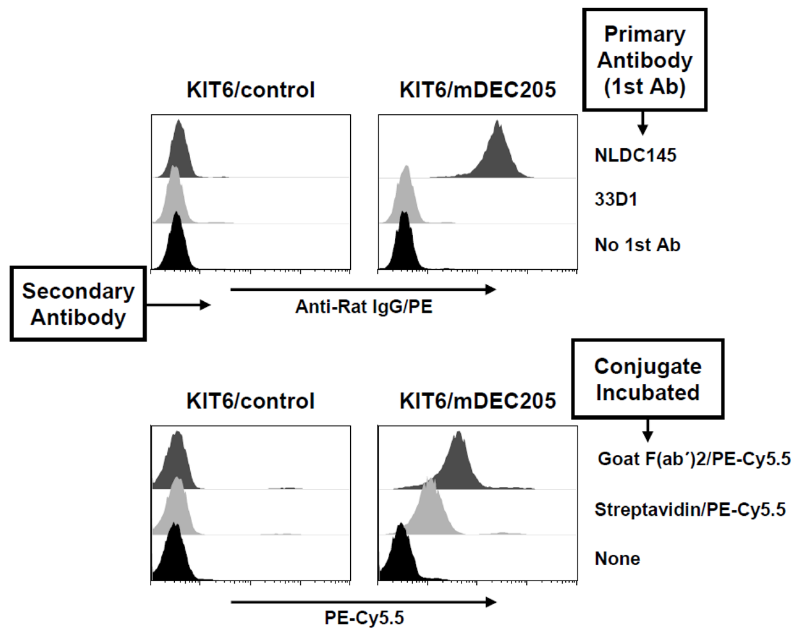

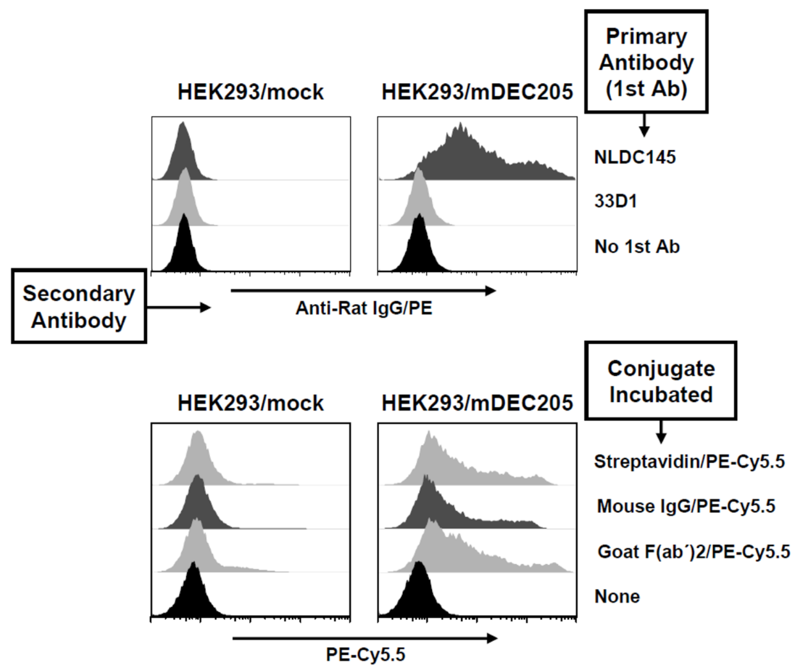

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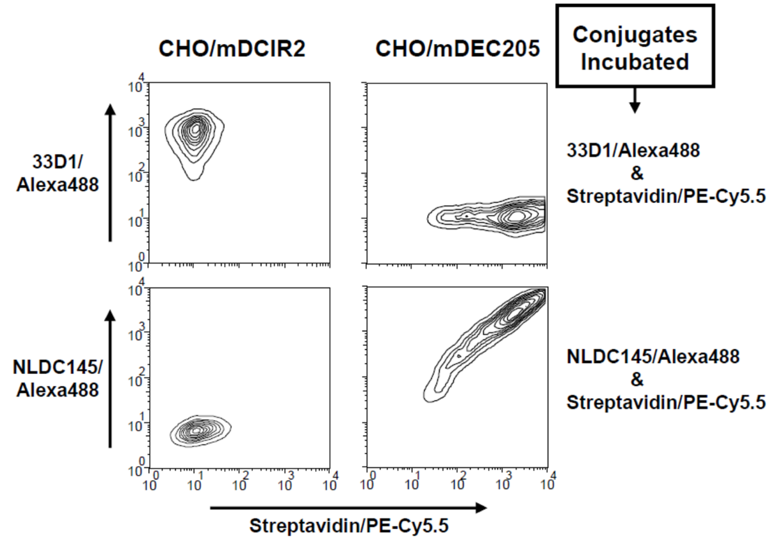

Fig 2. Nonspecific binding of PE-Cy5.5 conjugates in correlation with the mDEC205 expression on cell surface

(A) [upper panels] KIT6 T-cell hybridoma cells stably expressing MHC I Kb (KIT6/control) or mDEC205 (KIT6/mDEC205) were incubated with/without the primary antibody supernatant of NLDC145 or 33D1, followed by wash and incubation with PE-conjugated secondary anti-rat IgG at 0.25 μg/ml for flow cytometry. [lower panels] KIT6/control and KIT6/mDEC205 cells were incubated with/without each PE-Cy5.5 conjugate at 0.25 μg/ml, followed by flow cytometry.

(B) [upper panels] HEK293 cells transfected transiently with the expression vector encoding no insert (HEK293/mock) or mDEC205 (HEK293/mDEC205) were incubated with/without the primary antibody supernatant of NLDC145 or 33D1, followed by wash and incubation with PE-conjugated secondary anti-rat IgG at 0.25 μg/ml for flow cytometry. [lower panels] HEK293/mock and HEK293/mDEC205 cells were incubated with/without each PE-Cy5.5 conjugate at 0.5 μg/ml, followed by flow cytometry.

(C) CHO/mDCIR2 (left panels) or CHO/mDEC205 (right panels) cells were incubated with a blend of Alexa488-conjugated 33D1 and PE-Cy5.5-conjugated streptavidin (upper panels) or with a blend of Alexa488-conjugated NLDC145 and PE-Cy5.5-conjugated streptavidin (lower panels) for flow cytometry. The concentration of each fluorochrome conjugate in the blend was 5 μg/ml.

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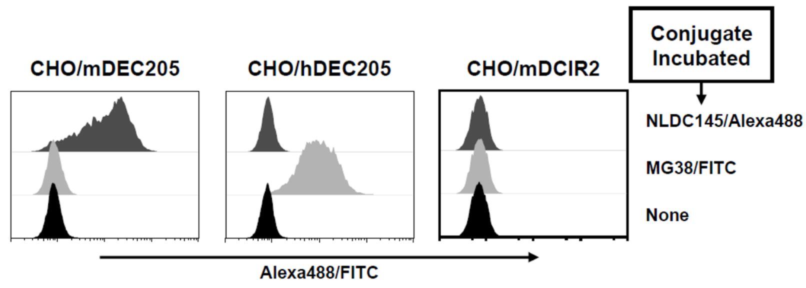

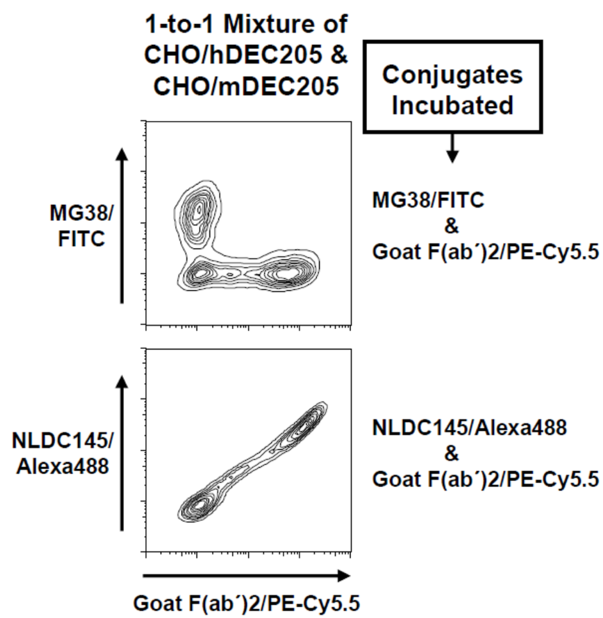

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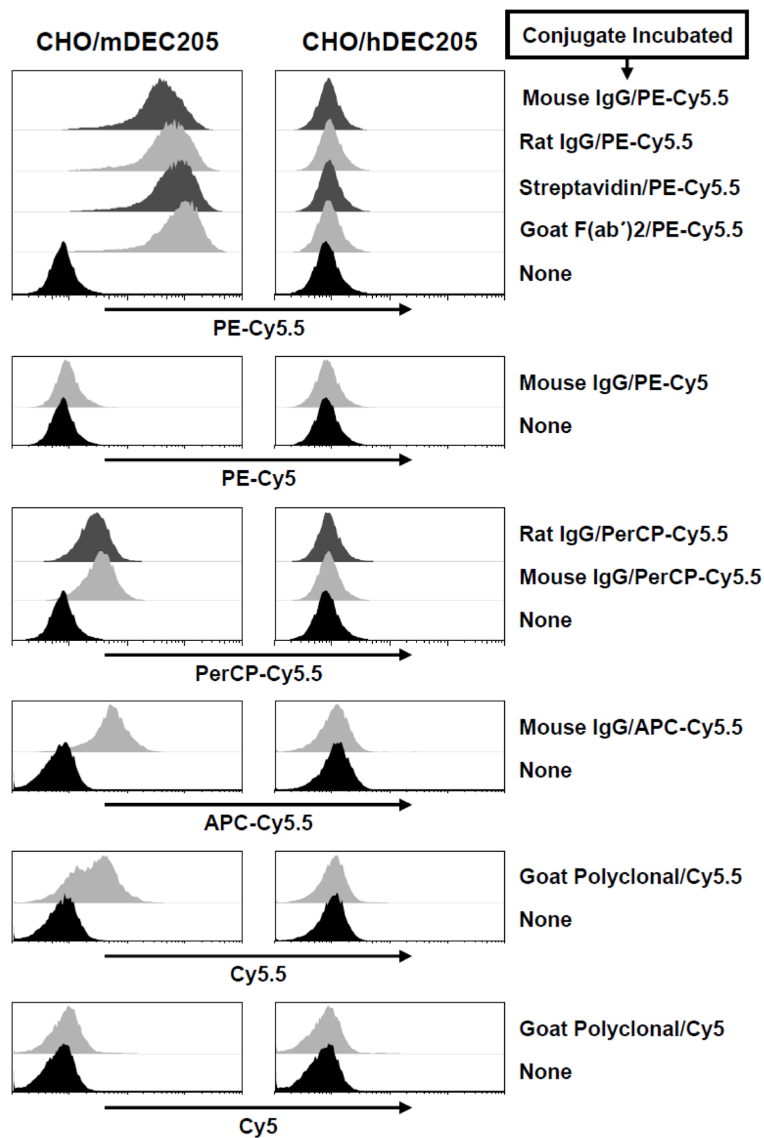

Fig 3. PE-Cy5.5 conjugates do not bind nonspecifically to hDEC205-positive cells

(A) CHO/mDEC205, CHO/hDEC205, and CHO/mDCIR2 cells were incubated with/without Alexa488-conjugated NLDC145 (anti-mDEC205; 0.5 μg/ml) or FITC-conjugated MG38 (anti-hDEC205; 5 μg/ml) for flow cytometry.

(B) An equal number of CHO/mDEC205 and CHO/hDEC205 cells were intermixed before the incubation with a blend of FITC-conjugated MG38 and PE-Cy5.5-conjugated goat F(ab′)2 (upper panel) or with a blend of Alexa488-conjugated NLDC145 and PE-Cy5.5-conjugated goat F(ab′)2 (lower panel) for flow cytometry. The concentration of each fluorochrome conjugate in the blend was 5 μg/ml for MG38/FITC or 0.5 μg/ml for NLDC145/Alexa488 and goat F(ab′)2/PE-Cy5.5.

(C) CHO/mDEC205 and CHO/hDEC205 cells were incubated with/without each fluorochrome conjugate at 0.5 μg/ml, except for the incubation with goat polyclonal/Cy5.5 and goat polyclonal/Cy5 at 2 μg/ml, followed by flow cytometry.

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