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Short communication

PD-L2 is expressed on activated human T cells and regulates their function

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ABSTRACT

T-cell activation and proliferation are regulated by cosignaling adhesion molecules involved in positive or negative signals. Programmed death (PD)-1 is one of immune inhibitory molecules that is expressed in activated T cells and is a promising target for immunotherapy. Both PD-1 ligands, PD-L1 and PD-L2 are expressed on antigen presenting cells (APCs) involved in the dialogue between a T cell and an APC. Here, we analysed the expression of these ligands, especially for PD-L2, on T cells. PD-L2 appears to be expressed on activated CD4 and CD8T cell subsets. Moreover, as PD-1 molecule, PD-L2 engagement at the surface of T cells is able to down-modulate cytokine production and cell proliferation. These observations indicate that PD-L2 is expressed following activation and is involved in the regulation of T cell function, highlighting the level of complexity in the T cell cosignaling network.

1. Introduction

T cell activation requires two signals that are delivered by antigen presenting cells (APCs). The first signal is induced by the T cell receptor (TcR) during antigen recognition. The second signal or costimulatory signal, is provided by adhesion molecules on APCs that bind to costimulatory receptors on T cells. The best well-characterized T cell costimulatory pathway involves the CD28 molecule engaged by two different ligands, B7-1 and B7-2 expressed on APCs (Greenwald et al., 2005). The discovery of additional members of the B7:CD28 family has revealed new costimulatory pathways that provide positive and/or negative second signals to effector T cells. Another «ménage à trois» has been identify corresponding to Programmed death 1 (PD-1) receptor on activated T cells and its two ligands, Programmed death ligand 1 (PD-L1) and Programmed death ligand 2 (PD-L2) on APCs (Greenwald et al., 2005). PD-1 has an important inhibitory function on activated lymphocytes by regulating the immune homeostasis and the maintenance of peripheral tolerance through its interaction with PD-L1 and/or PD-L2.

This T cell costimulatory panorama is even more complex as the ligands of the CD28 or PD-1 cosignaling molecules, normally expressed on APCs, can be also detected on T cells. B7-2 is constitutively expressed on some resting T cells, whereas B7-1 is not present at the resting stage (Taylor et al., 2004). Both B7-1 and B7-2 can be up-regulated on murine or human T cells (Sansom and Hall, 1993; Taylor et al., 2004) and deliver signals into T cells (Paust et al., 2004). PD-L1 is found constitutively expressed on murine T cells and is further up-regulated upon TcR stimulation (Yamazaki et al., 2002), whereas PD-L1 expression is inducible on human T cells (Dong et al., 2003). Autoantibodies against PD-L1 have been detected in sera from patients with rheumatoid arthritis. After immobilization, these antibodies stimulated cytokine production and T cell proliferation (Dong et al., 2003).

Thus, among the CD28/PD-1 ligands, only PD-L2 expression has not been investigated in human T cells. Here, we generate a new monoclonal antibody (mAb) against human PD-L2 and further analyze the PD-L2 protein expression on T cell subsets under different stimulation conditions. As PD-L2 appears to be inducible on $CD4^+$ and $CD8^+$ T cells, we further analyze the effects of PD-L2 mAbs using artificial APCs on activated T cells by cell proliferation and cytokine detection assays.

2. Materials and methods

2.1. Generation of anti-human PD-L2 mAbs

Female BALB/c mice were immunized by i.p. injection with 10 μg of human extracellular region of PD-L2/Ig fusion protein

Abbreviations: PD-1, programmed death 1; PD-L, programmed death 1 ligand; APCs, antigen presenting cells; TcR, T cell receptor; mAb, monoclonal antibody.

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with Freund adjuvant. PD-L2 mAbs were generated as previously described (Ghiotto et al., 2010; Serriari et al., 2010). The hybridoma supernatants were screened by cell surface staining of human PD-L2-transfected COS cells (Fig. S1A) and validated with a commercially available PD-L2 mAb (clone 24F.10C12, BioLegend) (Fig. S1B). PD-L2-326.35 clone (mouse, IgG1) was selected as reagent for FACS analysis and functional studies.

2.2. Cells and reagents

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors provided by Etablissement Français du Sang (EFS-Marseille-France). $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs by T cell negative isolation Kit (Miltenyi Biotec) and cultured in RPMI 1640 (GIBCO, Invitrogen) containing 10% foetal calf serum (FCS; Eurobio, Biotechnology). For FACS analysis, several fluorescent dye-coupled mAbs are used: CD4 FITC, CD8 PC5, CD3 PC7 and CD25 PE (BD Biosciences), CD25 FITC, TCRVβ8 PE and CD69 FITC (Beckman Coulter Immunotech). In addition, mAbs recognizing PD-1 (clone PD-1.6.4) and PD-L1 (clones PD-L1.1.8 and PD-L1.3) have already been described elsewhere (Ghiotto et al.; Serriari et al.). Staphylococcus Enterotoxin E (SEE) superantigen was purchased from Toxin Technology Inc. (Sarasota, FL, USA) and Cyclosporin A (CsA) was purchased from Novartis.

2.3. T cell stimulation

$CD4^+$ or $CD8^+$ T cells (1.5×10^5 cells/well) were activated in 96-well, flat-bottom plates (Costar, Cambridge, MA) coated with 1 μg/ml anti-CD3 (OKT3) and 2 μg/ml of soluble anti-CD28 (CD28.2) or with 5 ng/ml of SEE. T cells were also stimulated with artificial APCs (aAPC) comprised of magnetic beads (Dynabeads M-450 Epoxy, Dynal Biotech) coated with the following mAbs: anti-CD3 (OKT3), anti-CD28 (CD28.2), and/or various concentrations of anti-human PD-1, anti-human PD-L1 (clone PD-L1.1.8) or anti-human PD-L2. The amount of protein was kept constant at 20 μg/ml by the addition of control IgG1. As previously described (Serriari et al., 2010), these aAPCs were coated with suboptimal CD3 mAb (5%), suboptimal levels of CD28 mAb (10%), and either IgG1 Ab (CD3/28/IgG1), PD-1 mAb (CD3/28/PD-1 + IgG1), PD-L1 mAb (CD3/28/PD-L1 + IgG1), PD-L2 mAb (CD3/28/PD-L2 + IgG1), constituting the remaining 85% of protein added to the bead. T cells (1.5×10^5 cells/well) were stimulated in round-bottom 96-well plates with a cell/bead ratio at 1:1. In the experiments shown in Figs. 2A and 3C, a cell/bead ratio at 3:1 has been used.

2.4. Flow cytometry (FACS) analysis

To facilitate PD-1 ligands cell surface staining, PD-L1 mAb (clone PD-L1.1.8) was conjugated with Alexa fluor 488 and PD-L2 mAb (clone PD-L2-326.35) was conjugated with Alexa fluor 647 using a commercially available kit (Invitrogen, Paisley, UK). CD25 and CD69 expressions were used as activation control. Dead cells were detected using 7AAD Fixable Dead Cell Stain Kit (Invitrogen). Cells were analysed for expression kinetics of PD-L1 and PD-L2 on FACSCANTO flow cytometer (BD Immunocytometry Systems). Data were analysed by FlowJo software (Tree Star).

2.5. T cell assays

To determine the production of cytokines, cell-free supernatants were collected at 48 h and assayed for IL-2, IFN-γ and IL-10 by ELISA using OptEIA kits (BD Biosciences) according to the manufacturer's instructions. T cells were activated for 5 days in triplicate, cell proliferation was measured by ^{3}H-thymidine incorporation for the last 18 h or cell division was calculated by carboxyfluorescein diacetate succinimidyl diester (CFSE) dilution via FACS analysis.

To analyze PD-L2 transcripts expression, total RNA was isolated with Trizol (Invitrogen) for qRT-PCR analysis at 0, 16, and 24 h for CD3 + CD28 activated-$CD4^+$ T cells. The relative expression of PD-L1 and PD-L2 to GADPH transcripts was assayed in ABI 7500 real time PCR machine (Applied Biosystems, Inc). Primer and FAM-labelled probes for PD-L1, PD-L2 and GADPH, were purchased from Applied Biosystems, Inc.

2.6. Statistical analysis

All data were analysed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA). Comparisons were made between different conditions of stimulation. The Wilcoxon matched pairs test was used for the assays. The comparisons were made between treated with CsA or untreated cells (Fig. S2B). Differences were considered as statistically significant when $p < 0.05$.

3. Results

3.1. Inducible expression of PD-L2 in human primary T cells

PD-1 ligands are expressed on APCs. Expression of the first ligand, PD-L1 has been also reported on T cells (Dong et al., 2003; Yamazaki et al., 2002). Here, the potential expression of the second ligand, PD-L2 is investigated on human T cell subsets. We generate a PD-L2 mAb that is used to stain T cells isolated from blood of volunteer donors (see Section 2 and Fig. S1A). Isolated $CD4^+$ and $CD8^+$ T cell subsets were stimulated with plate-bound anti-CD3 plus soluble anti-CD28. Cell aliquots were harvested each day along a period of four days and stained with the PD-L2 mAb for FACS analysis (Fig. 1A). As previously described (Brown et al., 2003), PD-L2 is not expressed in resting $CD4^+$ T cells. It is also the case for resting $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells both demonstrated a progressive increase in the intensity of PD-L2 expression that started after 48 h, especially for $CD8^+$ T cells. The T cell activation status is controlled by the expression of a well-known activation marker, CD25 both on $CD4^+$ and $CD8^+$ T cells (Fig. 1B). Similar PD-L2 staining is detected on activated T cells using a commercially available PD-L2 mAb (Fig. S1B). Moreover, the PD-L2 mRNA expression on CD3 plus CD28-activated T cells is analysed by qRT-PCR by using specific primer for PD-L2 (Fig. S2B), showing that PD-L2 transcript levels are increased upon CD3 plus CD28 costimulation on $CD4^+$ T cells. PD-L1 transcript detection is used as a positive control, PD-L1 transcripts were detected at the earliest time point analysed whereas PD-L2 transcripts were detected only after 24 h of stimulation.

Other kinds of T cell activation have been used as T cells are expressing a very low amount of PD-L2 molecules in mixed leukocyte reaction (data not shown) or using the staphylococcus enterotoxin E (SEE) as a superantigen (SAg). This SAg induces the strong activation of the TcR-$V\beta8^+$ T cells (Garcon et al., 2004). The expression of both PD-1 ligands, PD-L1 and PD-L2 on $V\beta8^+$ T cell subpopulation are analysed by flow cytometry. PD-Ll and PD-L2 were detected after 48 and 96 h upon SEE stimulation respectively (Fig. 1C). Dot plot TCR-Vβ8/PD-L2 is showing that the T cell subpopulation that is growing upon 2 days of SEE stimulation is expressing both TCR-Vβ8 and PD-L2 (Fig. 1D).

Altogether, these results are showing that PD-L2, as PD-L1 is expressed on human activated T cells.

3.2. The PD-L2 engagement inhibits the T-cell cytokine production

Whether PD-L1 and PD-L2 have a stimulatory or inhibitory function is a matter of debate, and could be partially dependent on the model system used (Kuipers et al., 2006; Van Keulen et al., 2006).