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Tumor PD-L1 co-stimulates primary human CD8$^+$ cytotoxic T cells modified to express a PD1:CD28 chimeric receptor

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ABSTRACT

Tumors exploit immunoregulatory checkpoints that serve to attenuate T cell responses as a means of circumventing immunologic rejection. Programmed death ligand 1 (PD-L1) is a negative regulator of T cell function and is frequently expressed by solid tumors. By engaging programmed death 1 (PD-1) on activated T cells, PD-L1$^+$ tumors directly render tumor-specific T cells, including adoptively transferred T cells, functionally exhausted. As a strategy to overcome tumor PD-L1 effects on adoptively transferred T cells, we sought to convert PD-1 to a T cell costimulatory receptor by exchanging its transmembrane and cytoplasmic tail with that of CD28. Rather than becoming exhausted upon engagement of PD-L1$^+$ tumors, we hypothesized that CD8$^+$ cytotoxic T lymphocytes (CTL) genetically modified to express this PD1:CD28 chimera would exhibit enhanced functional attributes. Here we show that cell surface expressed PD1:CD28 retains the capacity to bind PD-L1 resulting in T cell costimulation as evidenced by increased levels of ERK phosphorylation, augmentation of cytokine secretion, increased proliferative capacity, and enhanced expression of effector molecule Granzyme B. We provide evidence that this chimera could serve as a novel engineering strategy to overcome PD-L1 mediated immunosuppression.
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1. Introduction

Tumors employ a variety of countermeasures that impair the functional integrity of tumor specific T cells; these countermeasures include the expression of ligands for inhibitory receptors expressed on T cells (Dong et al., 2002; Gajewski et al., 2006a,b; Iwai et al., 2002) and the lack of ligands for T cell costimulatory receptors (Anderson et al., 2007; Brown et al., 1998; Denfeld et al., 1995; Groh et al., 2002; Tatsumi et al., 1997). Indeed, one inhibitory receptor ligand, PD-L1, has specifically been identified as a poor prognostic indicator for several tumor types including myeloma, pancreatic, renal, ovarian, and breast cancers (Ghebeh et al., 2006; Hamanishi et al., 2007; Hino et al., 2010; Nomi et al., 2007; Thompson et al., 2006). PD-L1 interacts with PD-1, a monomeric inhibitory receptor expressed on the surface of activated T cells (Freeman et al., 2000). PD-L1 ligation of PD-1 results in the activation of phosphatase SHP-1 and leads to inhibition of TCR signaling through decreased CD3ξ and Zap-70 phosphorylation, as well as by inhibiting CD28 signaling through inhibition of PI3K and Akt activation (Parry et al., 2005; Sheppard et al., 2004). Within the tumor microenvironment, the PD-1:PD-L1 interaction results in T cell exhaustion manifested as the step-wise loss of cytokine secretion (IL-2 > TNF-α > INF-γ), cytolytic activity, proliferative capacity, and finally T cell apoptosis (Freeman et al., 2000, 2006; Hirano et al., 2005; Wintterle et al., 2003). Furthermore, PD-1 or PD-L1 blockade by monoclonal antibodies can result in the restoration of T cell function and improved anti-tumor efficacy of adoptively transferred T cells *in vivo* (Freeman et al., 2000; Hirano et al., 2005; Strome et al., 2003; Wintterle et al., 2003).

Apart from inhibitory ligand expression in the tumor microenvironment, full T cell activation requires not only antigenic stimulation *via* the T cell receptor (TCR; signal 1), but also a costimulatory signal (signal 2) to prevent functional anergy (Harding et al., 1992). CD28 is the primary costimulatory receptor for CD4$^+$ T_H and naïve/memory CD8$^+$ T cells, while activated CD8$^+$ effector T cells down regulate CD28 and instead can be costimulated through NKG2D (Rajasekaran et al., 2010). Within the tumor microenvironment, expression of CD28 ligands (B7-1 and -2) are often absent (Anderson et al., 2007; Brown et al., 1998; Denfeld et al., 1995; Tatsumi et al., 1997), while NKG2D ligands MICA and MICB are shed from tumor cells (Groh et al., 2002), limiting costimulation of anti-tumor T cells. The inability of responding

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T cells to be costimulated once in the tumor microenvironment results in their acquisition of functional anergy and deletion (Gajewski et al., 2006a,b), whereas forced expression of transgenic costimulatory ligands by tumor cells in animal models corrects this deficit, resulting in enhanced T cell function and tumor control (Townsend and Allison, 1993). In addition, expressing a CD28 cDNA in effector $CD8^+$ CTL, following downregulation of endogenous CD28 expression as a consequence of TCR activation, can reconstitute costimulatory responsiveness to B7-1 and -2 (Topp et al., 2003). Here we decided to test whether a PD1:CD28 chimeric receptor converts tumor PD-L1 to a ligand that transmits a CD28 costimulatory signal to $CD8^+$ effector CTL, thereby augmenting their anti-tumor effector function.

2. Materials and methods

2.1. cDNA constructs and lentivirus expression vectors

PD1:CD28_epHIV7 contains a truncated extracellular PD-1 (AA1-155), derived from PD-1 cDNA (Origene, Rockville, MD), and transmembrane and cytoplasmic domains of CD28 (AA141-220), fused by PCR at the transmembrane proximal extracellular CD28 cysteine (AA141) required for homodimerization (Young et al., 1994). PDL1-eGFP_epHIV7 contains the full length PD-L1, cloned from U251T cDNA, fused to an enhanced GFP gene (Clontech, Mountain View, CA). OKT3op-2A-eGFP-ffluc_pHIV7 contains the codon optimized OKT3 sequence, which is the scFv of the anti-human-CD3ε mAb fused to huIgG4 Fc (gift from Andrew Raubitschek, City of Hope National Medical Center (COHNMC)) that is in turn fused to the transmembrane domain of huCD4 and linked *via* a 2A peptide sequence to an enhanced GFP gene (Clontech), and the firefly luciferase gene (Invivogen, San Diego, CA). The epHIV7 lentiviral vector was generated from the pHIV7 vector backbone (gift of Jiing Kuan Yee COHNMC). All constructs and construction associated PCR primer sequences are available upon request.

2.2. Cell lines and cultures

Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) from heparinized peripheral blood obtained from consented healthy donors participating in Internal Review Board protocols (COHNMC). $CD8^+$ cells were isolated from PBMC by AutoMACS™ (Miltenyi Biotec Inc, Auburn, CA) immunomagnetic sorting *via* anti-CD4 (BD Biosciences, San Jose, CA) depletion following the manufacturer's protocol. T cells stimulated with CD3/CD28 beads (Dynal, Oslo, Norway) in a bead to cell ratio 3:1 were transduced (MOI 5) with PD1:CD28_epHIV7 as previously described (Chinnasamy et al., 2000). Transduction efficiency without sorting was approximately 30% and enrichment was performed using AutoMACS™ immunomagnetic sorting using anti-PD1 (eBioscience, San Diego, CA) per the manufacturer's protocol. AutoMACS sorting using anti-PD1 did not appear to result in stimulation/activation of T cells, as OKT3-mediated stimulation was required for PD1:CD28 chimera signaling (*e.g.*, see Section 3.3).

T cells were maintained in RPMI-1640 with 10% heat inactivated fetal calf serum, 25 mmol/L HEPES-BSS, 2 mmol/L L-glutamine, and 25 or 50 U/ml IL-2. Every fourteen days T cells underwent rapid expansion method (REM) as described in Jensen et al. (2000). All assays using primary human T cells were performed between days 14–17 of the sixth stimulation cycle.

H9 cells (provided by Lijing Li, COHNMC) were transduced as described above. Transduction efficiency was greater than 70% and, therefore, sorting was not performed. H9 cells were maintained in RPMI supplemented as described above without the addition of exogenous IL-2.

U293T cells are a transformed cell line developed from human embryonic kidney cells. These cells are PD-L1, B7-1, and B7-2 negative, but are positive for NKG2D ligands. In some cases U293T cells were lentivirally transduced with OKT3op-2A-eGFP-ffluc_pHIV7 and/or PDL1-eGFP_epHIV7 at MOI 1.6 and 10 respectively. U87 and U251T are primary human glioblastoma cell lines that are negative for CD28 ligands, but positive for PD-L1. In some cases U87 and U251T cells were lentivirally transduced with OKT3op-2A-eGFP-ffluc_pHIV7 at MOI 10 and 2.5 respectively. GFP-expressing U87 cells were sort-purified using a MoFlo MLS (Dako-Cytomation, Fort Collins, CO). U293T, U87, and U251T cells were maintained in DMEM supplemented as described above.

2.3. Flow cytometric analysis

Fluorochrome-conjugated isotype controls, anti-CD8, anti-CD28, anti-CD80, anti-CD86, anti-NKG2D, and streptavidin were purchased from BD Biosciences. Fluorochrome-conjugated anti-B7H1 (clone M1H1) and anti-PD1 (clone M1H4) were purchased from Ebioscience. Biotinylated anti-Fc was purchased from Jackson Immunoresearch (West Grove, PA). In order to assess PD-L1 binding to the PD1:CD28 chimera and NKG2D ligand binding, 10 μg/ml recombinant human B7H1.Fc or NKG2D.Fc (R&D Systems, Minneapolis, MN) was used followed by biotinylated anti-huFc and fluorochrome-conjugated streptavidin, as is also used for the detection of OKT3. All antibodies were used according to the manufacturer's instructions. Flow cytometric data acquisition was performed on a FACScalibur (BD Biosciences). Axes for all FACS plots are log scale and the percentage of positively expressing cells within a region was calculated *via* FCS Express 3 (De Novo Software, Los Angeles, CA).

2.4. Antibody stimulation and Western blotting

H9 cells and CTL were stimulated with 1 μg/ml anti-human CD3 monoclonal antibody (OKT3) (Orthobiotech, Raritan, NJ) + 10 μg/ml goat anti mouse (GaM) Fc antibody (Jackson Immunoresearch) and/or 10 μg/ml recombinant human PD-L1 Fc chimera (B7H1.Fc) (R&D Systems) + 10 μg/ml goat anti human (GaH) Fc antibody (Jackson Immunoresearch) for 0 or 15 min. In alternate samples, CTL were stimulated 1:1 with U293T, $OKT3^+$ U293T, or $OKT3^+$PD-$L1^+$ U293T cells for 0 or 15 min. Cells were lysed in 1% Triton lysis buffer supplemented with phosphatase inhibitor cocktail II (Sigma–Aldrich Corp., St. Louis, MO). Western blots were probed with anti-phospho ERK, anti-ERK, anti-phospho-AKT, and anti-AKT antibodies (Cell Signaling Technology, Danvers, MA), anti-B7-H1 (R&D Systems), or anti-βactin (Rockland Immunochemicals, Gilbertsville, PA) as per the manufacturer's instructions. Blots were imaged on the Odyssey Infrared Imager (LI-COR, Lincoln, NE) and band intensities were quantified using Odyssey v2.0 software (LI-COR).

2.5. Cytokine assays

Responders (10^6) were cultured at a 1:1 ratio with tumor stimulators in 2 ml in a 12 well plate with 0 U/ml IL-2. For intracellular staining assays, following 15–18 h of co-culture, Brefeldin A (BD Biosciences) was added for an additional 6 h of culture. Cells were then collected and stained with fluorochrome-conjugated anti-CD8, anti-IFNγ, anti-IL2 (BD Biosciences), or anti-TNF-α (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. In similar assays, supernatants were collected after co-culture and examined by cytometric bead array (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Output data were analyzed *via* the Bio-Plex Manager 4.0 (Bio-Rad).

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