International Immunopharmacology 31 (2016) 248–256

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

A fully human monoclonal antibody targeting PD-L1 with potent anti-tumor activity

Yan Luan [a,b], Dafei Chai [a], Jianjian Peng [a], Shuli Ma [a], Min Wang [a], Hui Ma [a], Xiang Li [a], Shilong Fu [a], Xiaolong Pan [a], Xiaoxiao Wang [d], Songbing Qin [c,*], Ting Xu [a,d,**]

[a] DingFu Biotarget Co. Ltd., Suzhou, Jiangsu 215125, PR China
[b] Chinese Academy of Sciences Key Laboratory for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China
[c] Department of Radiotherapy, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, PR China
[d] Alphamab Co. Ltd., Suzhou, Jiangsu 215125, PR China

ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 7 December 2015
Accepted 29 December 2015
Available online xxxx

Keywords:
Monoclonal antibody
PD-L1
B60-55
Tumor
Therapeutic agent

ABSTRACT

Background: Programmed cell death ligand-1 (PD-L1) with its receptor PD-1 pathway is overactivated in many tumors. Inhibiting the interaction of PD-L1 and PD-1 is an attractive strategy to restore tumor-specific T cell immunity for tumor therapy.
Methods: A fully human anti-PD-L1 monoclonal antibody (mAb) B60-55 was identified by yeast surface display. The affinity, specificity, activity, and efficacy of mAb B60-55 were investigated in vitro or in vivo.
Results: mAb B60-55 (purity >99%) could bind to PD-L1 that is expressed on HEK293 cells with a dissociation constant of 0.2 nM, and specifically bind to human or cynomolgus macaque PD-L1 without a cross-reaction with murine PD-L1. Moreover, mAb B60-55 is an antagonistic antibody, which can block PD-L1 binding to its receptors, including PD-1 (PDCD1) and B7.1 (CD80). In vitro assays demonstrated the ability of mAb B60-55 to enhance T cell responses and cytokine production in the mixed lymphocyte reaction. In vivo studies showed that administration of mAb B60-55 exhibited a potent antitumor activity toward tumor cell carcinoma xenograft, with a mean half-life of 177.9 h in cynomolgus monkeys.
Conclusion: mAb B60-55 is a potential candidate for clinical development in cancer treatment.

1. Introduction

Programmed death 1 (PD-1; CD279) is a member of CD28 family that is overexpressed by activated T cells, B cells, dendritic cells, macrophages, and myeloid cells [1–4]. PD-1 encounters with its ligand programmed cell death ligand-1 (PD-L1; B7-H1 or CD274), which is expressed by tumor cells and immune cells [5]. PD-L1 is a type I transmembrane protein that is approximately 53 kDa, and it contains two extracellular Ig domains, which include an N-terminal V-type domain followed by a C-type domain [6]. Studies demonstrated that PD-L1 expression in tumor tissues can be used as an independent factor to evaluate the prognosis of gastric carcinoma [5,7]. The PD-1/PD-L1 molecular pathway is a negative regulator of co-signaling pathway that functions as a cellular checkpoint to suppress inflammatory and immune responses against cancer [8–10]. PD-1/PD-L1 functions through several alternative mechanisms, which include inducing the exhaustion of tumor infiltrating T lymphocytes, stimulating secretion of immune repressive cytokines into the tumor microenvironment, stimulating repressive regulatory T cell function, and protecting PD-L1-expressing tumor cells from lysis by tumor cell specific cytotoxic T cells [11–13]. Modulation of the immune responses involved in immune-mediated disorders can be accomplished by manipulating the PD-1/PD-L1 pathway [14]. Therefore, inhibition of the interaction between PD-1 and PD-L1 can enhance T-cell responses and mediate preclinical antitumor activity.

PD-1/PD-L1 interaction is considered a negative regulator of T-cell effector mechanisms, and this interaction limits the immune responses against cancer [15,16]. Monoclonal antibodies (mAbs) that antagonize either PD-1 or PD-L1 are currently in various stages of development for cancer treatment, in which recent human trials showed promising results in advanced, treatment-refractory diseases [17,18]. Remarkable results observed in clinical trials made the US Food and Drug Administration approve the use of pembrolizumab and nivolumab (both anti-PD-1 antibodies) to treat advanced melanoma in late 2014 and to treat non-small cell lung carcinoma in 2015 [16]. Anti-PD-L1 mAbs, MEDI4736, and MPDL3280A are in the late-stage multicenter clinical trial studies for different types of advanced cancers, such as melanoma, non-small cell lung carcinoma, renal cell carcinoma, and ovarian cancer [19–21]. They have promoted durable tumor regression and prolonged stabilization of disease in patients [14]. Based on these observations,

* Corresponding author.
** Correspondence to: T. Xu, DingFu Biotarget Co. Ltd., Suzhou, Jiangsu 215125, PR China.
E-mail addresses: qin92244@163.com (S. Qin), tingxu@dingfubio.com (T. Xu).

http://dx.doi.org/10.1016/j.intimp.2015.12.039
1567-5769/

anti-PD-L1 antibodies can be used therapeutically to enhance antitumor immune responses in cancer patients.

Yeast surface display is a powerful tool for antibody screening and further improvement on affinity, specificity, and stability [22]. Antibodies of interest are expressed on yeast surface, and its protein properties, such as stability and affinity, can be quantitatively measured by using fluorescently labeled reagents and flow cytometry [23]. The method for isolation of novel antibodies against specific antigens from a non-immune human antibody library is well established. In the present study, we developed three novel fully human IgG1 anti-PD-L1 mAbs (BII61-62, B60-55, and B50-6) by yeast surface display. The mAb B60-55 exhibited specific binding to PD-L1 with a higher affinity than the other two mAbs. In addition, mAb B60-55 could enhance T cell activation by blocking PD1 binding to PD-L1 and exhibited strong antitumor effects in preclinical models. Our study showed that B60-55 is a potent antagonist of PD-L1 with significant antitumor activity in mouse models, and it is a candidate for clinical trials.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female inbred C57BL/6 or NOD/SCID mice were obtained from the Experimental Animal Centre of Chinese Academy of Science (Shanghai, China) and housed in a specific pathogen-free room under controlled temperature and humidity. This study was performed in strict accordance with the recommendations provided in the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China, 1998). The protocols for mouse studies and pharmacokinetic (PK) study in cynomolgus monkey were approved by the local ethics committee.

2.2. Cell lines and reagents

Adherent human embryonic kidney HEK293 cells (Invitrogen R79007), murine colon cancer cell line MC38 (ATCC), and human melanoma cell line A375 (ATCC, CRL-1619™), or A375 stable cell line highly expressing PD-L1 (prepared in house) were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco Invitrogen). HEK293 cells that express human PD-L1 (hPD-L1 cells), human PD-1 (hPD-1 cells), and murine PD-L1 (mPD-L1 cells) were generated in our laboratory and maintained with RPMI 1640 culture medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Yeast strain EBY100 (GAL1-AGA1::URA3 ura3-52 trp1 leu21 his3200 pep4:HIS2 prb11.6R can1 GAL) was maintained in YPD broth (Difco).

2.3. Expression protein of hPD-L1 or hPD-1

DNA encoding residues 19–238 (hPD-L1) and 21–170 (hPD-1) were fused to human Fc fragment by overlapping PCR. The overlapping PCR product were cloned into a pcDNA3.1 expression vector and used for transfection and protein expression. hPD-L1-Fc and hPD-1-Fc fusion proteins were secreted in the media by transfecting HEK293 cells as described elsewhere [24] and were purified by Protein A affinity chromatography (GE Healthcare). The homogeneity and purity of the protein preparations were verified by SDS-PAGE and SEC-HPLC. Protein concentrations were measured by A280 assay. hPD-L1-Fc or hPD-1-Fc fusion protein was labeled with biotin by standard protocol.

2.4. Library construction

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) of 150 healthy donors and reverse transcribed into cDNA by using transcriptor first-strand cDNA synthesis kit (Thermo). The HuIgG, HuIgM, Huκ forward, and Huλ forward primers that were used for the gene-specific cDNA synthesis were based on Marks et al. [25]. The scFv yeast display vector pDFYD was generated from the vector pCT302 (Addgene). A BssHII cloning site was inserted at the 6029 bp site of the pCT302 by using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). 21-amino-acid linker (5′-CCATGGGGATCCGG TGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGTCGAC-3′) was inserted at the 5603 bp site of the pCT302 by overlap PCR. The primers were based on the method previously described [26] with slight modifications. Certain sequences were added to the 5′ end of the forward primers and 5′ end of the reverse primers (5′-GCCGGCTAGC-3′ for VH forward primers), (5′-GCCGCCATGG-3′ for all VH reverse primers), (5′-GCCGGTCGAC-3′ for all forward Vλ and Vκ primers), and (5′-GCCGGCGCGC-3′ for all reverse Vλ and Vκ primers) to allow cloning into pDFYD vector. Kappa and lambda light-chain genes were gel purified with the QIAquick Gel Extraction Kit (Qiagen), pooled in an equal ratio, digested with the restriction enzymes BssHII and SalI, and ligated into the vector pDFYD. After ethanolic precipitation, the ligation mixture was used to transform *Escherichia coli* strain by using Electro-Ten Blue (Stratagene). pDFYD plasmid DNA that contained the light-chain genes was isolated by using a Maxiprep kit (Qiagen) and digested with the restriction enzymes NheI and NcoI for ligation of the heavy-chain genes. The libraries were then linearized with BssHII, ethanol precipitated, and used to transform the yeast strain EBY100 by using the high-efficiency lithium acetate method. The size of the scFv libraries were approximately 1×10^8, which was estimated by standard plate count method. To increase antibody affinity, scFv sequence was randomly mutated by using error-prone PCR as previously described [27]. The error prone PCR products were ligated to pDFYD plasmid, and the library was constructed as described above.

2.5. Screening of mAb anti-hPD-L1

The yeast library with tenfold diversity was thawed, grown in SD-CAA media for 24 h at 30 °C, and then cultured in SG-CAA for 36 h at 20 °C in volumes appropriate for the library size. Sequential magnetic bead enrichment and flow-cytometric sorting strategy were developed to isolate antigen-specific scFvs from the full library. One hundred nanomolars PD-L1-Biotin and SA-Beads were used for the first round of magnetic selection, and one-hundred nanomolars PD-L1-Biotin and anti-Biotin-Beads were used for the second round of selection. The following two rounds of sorting were performed by using flow cytometry to sort out PD-L1 Fc antigen positive and myc positive cells, which indicate that complete antibody expression on yeast can bind target antigen. After the final round, yeasts were placed on SD-CAA plates and individual colonies were picked up for characterization. Thousands of single yeast colonies were screened by high throughput flow cytometry analysis, with PD-1-Fc-biotin protein as the negative control.

2.6. Expression and purification of antibodies

The scFv-coding genes from the identified yeast clone were overlapped with human Fc and then cloned into expression vector pcDNA3.1 by using NheI and NotI for scFv-Fc expression. To produce full length mAbs, the VH gene were cloned from the identified yeast clone and overlapped with the human IgG1 C-region gene containing three mutations to get rid of ADCC and CDC function. The VL genes were cloned from the identified yeast clone and overlapped with the human kappa or lambda C-region gene. The overlapped fragments were further cloned into pcDNA3.1 vector by using NheI and NotI separately.

The scFv-Fc fusion protein or fully mAb IgG protein were secreted in the media by transfected HEK293 cells as described previously, and purified by Protein A affinity chromatography (GE Healthcare). The homogeneity and purity of the protein preparations were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) and size