Molecular Immunology 77 (2016) 141–147

Contents lists available at ScienceDirect

Molecular Immunology

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

Generation and characterization of human B lymphocyte stimulator blocking monoclonal antibody

Weiliang Zhuang[a,1], Jianjun Zhang[b,1], Lili Pei[a], Shuping Fang[a], Honghao Liu[a], Ruixue Wang[b], Yunpeng Su[a,*]

[a] Novomab Biopharmaceuticals Inc., Nanjing 210042, China
[b] China Pharmaceutical University, Nanjing 210009, China

ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 23 July 2016
Accepted 2 August 2016
Available online 6 August 2016

Keywords:
Autoimmune
SLE
Blys
Antibody

ABSTRACT

The cytokine, B lymphocyte stimulator (Blys) is essential for activation and proliferation of B cells and is involved in the pathogenesis of B-cell mediated autoimmune diseases. Based on its essential activity, Blys may be a potential therapeutic target for human autoimmune diseases. In this article, we have described the development of a novel humanized anti-Blys antibody, NMB04, that binds with high affinity and specificity to both soluble and membrane bound Blys. This monoclonal antibody has the potential to block Blys binding to all its three receptors, TACI, BCMA and BR-3. Further *in vivo* studies revealed that NMB04 possessed more potent inhibitory activity against human Blys as compared to an existing antibody, Belimumab. Therefore, NMB04 may have potential as a therapeutic candidate targeting autoimmune diseases.

1. Introduction

B lymphocyte stimulator (Blys) also termed as BAFF, TALL-1, zTNF4 or THANK, is a co-stimulatory cytokine that belongs to the tumor necrosis factor (TNF) ligand superfamily. Blys plays an essential role in B-cell differentiation, proliferation, maturation and survival (Moore et al., 1999; Schneider et al., 1999; Shu and Hu, 1999; Mukhopadhyay et al., 1999; Gross et al., 2000). It is largely expressed by monocytes, macrophages, dendritic cells and activated T cells in a membrane-bound and soluble form. Both of these forms can actively promote the proliferation of B lymphocytes stimulated with anti-immunoglobulin M (Moore et al., 1999; Shu and Hu, 1999). Blys displays high affinity binding to three receptors expressed by B cells, transmembrane activator-1 and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA) and Blys receptor 3 (BR3 or BAFF-R). The TACI and BCMA receptors are also shared by cytokine APRIL, which is a homolog of Blys (Gross et al., 2000; Yan et al., 2001). Previous *in vitro* and *in vivo* studies have shown that Blys can promote, not only B cells proliferation and survival, but also increase serum immunoglobulin levels. In addition to co-stimulation of T cells, Blys also enhances the T cell dependent and independent humoral immune responses (Moore et al., 1999; Schneider et al., 1999; Do et al., 2000; Schneider, 2005). Furthermore transgenic mice overexpressing Blys developed autoimmune-like manifestations (Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999). Elevated levels of Blys have also been reported in murine models of systemic lupus erythematosus (SLE) (Gross et al., 2000) and in some patients with autoimmune diseases, including SLE (Cheema et al., 2001; Stohl et al., 2003; Zhang et al., 2001; Petri et al., 2008), rheumatoid arthritis (RA) (Cheema et al., 2001) and Sjogren's syndrome (SS) (Groom et al., 2002; Mariette et al., 2003). These prior studies have shown significant correlation between Blys serum levels and autoantibody levels. Interestingly, the neutralization of Blys by its antagonist TACI-Ig fusion protein, led to inhibition of the disease in mouse models of SLE and RA, and BAFF-deficient mice displayed dramatic reduction in the population of mature B cells and circulating immunoglobulin levels, which can be attributed to the blockage of B cell development (Gross et al., 2000; Gross et al., 2001; Schiemann et al., 2001).

The prior work suggests that Blys may be a promising therapeutic target for the treatment of B cell mediated autoimmune diseases. In 2011, Belimumab, an anti-Blys antibody was approved by the United States Food and Drug Administration (FDA) as the first biological drug for the treatment of SLE in more than 50 years (Stohl and Hilbert, 2012). In addition to phase 3 trials of Belimumab in SLE (Navarra et al., 2011), the safety and efficacy of Blys-targeting

* Corresponding author at: Novomab Biopharmaceuticals Inc. No. 699-18, Xuanwu Avenue, Xuanwu District, Nanjing, 210009, China.
E-mail address: suyunpeng@simcere.com (Y. Su).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.molimm.2016.08.002
0161-5890/

therapies have also been observed in other clinical trials of other two anti-Blys agents (Tabalumab and blisibimod) in autoimmune diseases, including SLE, RA and SS (Clinical, 2016a,b,c,d; Kamal and Khamashta, 2014). Despite this initial progress, still more research is required to develop a new BLYS blocking antibody for the treatment of severe SLE patients that have developed kidney and brain damage and were not part of the clinical trial of Belimumab (Navarra et al., 2011).

Thus in this study, we have attempted to generate a new antagonistic humanized monoclonal antibody against human Blys which has the potential to be used as an alternate therapy in place of belimumab.

2. Methods

2.1. Generation and screening of murine anti-Blys monoclonal antibodies (mAbs)

The anti-human Blys antibodies were generated by immunizing BalB/C female mice with recombinant human BlyS (Sino Biological Inc.10056-HNCH) following the established protocol. The generated hybridomas, secreting Blys-specific antibodies, were screened by ELISA and IM9 cell binding inhibition assays. Subsequently, the positive clones were further subcloned and were expanded in Hybridoma SFM medium (Gibco). For additional functional assays, monoclonal antibodies were purified from clonal supernatant by protein G affinity chromatography (Genscript, L00209).

2.2. Humanization and maturation of anti-Blys mAbs

The cDNAs from the hybridomas were amplified by RT-PCR. Next, the variable regions from the heavy (VH) and light chain (VL) of Blys antibody were obtained by PCR and sequencing. Mouse variable region genes were aligned against the human germ line database, and the closest human immunoglobulin germline sequences to the murine VL and VH were selected as the human donor frameworks. After CDR grafting as describing previously (Jones et al., 1985), humanized VL and VH were constructed in the form of scfv which were then used as PCR template to construct the scfv phage library. RGYW or AGY hot-spot motifs in the CDR were selected and CDR hot-spot libraries were constructed by site directed mutagenesis PCR. Affinity improvements were achieved after three to six rounds of panning against soluble human Blys. Affinity maturation clones were screened by IM9 cell binding inhibition assay, and selected clones were constructed and expressed as a full IgG for further identification.

2.3. IM9 cell binding inhibition assay

IM9 cells (ATCC) were immobilized onto poly-L-lysine coated 96-well plates at a density of 1×10^5cells/well and then blocked with 1% (w/v) BSA/PBS for one hour. The 50 μl antibody or scFv phage was mixed with 50 μl of biotinylated Blys (100 ng/ml) and added to 96-well plates containing IM9 cells for one hour. Plates were washed using PBS for three times, and the biotinylated Blys was detected *via* streptavidin-Delfia technology (PerkinElmer). For effective antibody identification, the IC_{50}values, based on the competition of each antibody to inhibit Blys binding to its receptor, were determined.

2.4. Murine splenocyte proliferation assay

The splenocytes isolated from the spleen of BalB/C mice were added to 96-well plate at a cell concentration of 1×10^6cells/well. Later, serial 2-fold antibody dilutions premixed with Blys (1.5 ng/ml final concentration) were added to each well. All substances were diluted in complete medium (RPMI 1640 with 10% fetal bovine serum containing 100 units/ml of penicillin, 100 g/ml of streptomycin, 2 mM L-glutamine, 50 μMmercaptoethanol and 5 μg/ml LPS).The plates were incubated for 72 h at 37 °C in a 5% CO2 incubator. The proliferation of cell was determined via CellTiter-Glo assay (Promega).

2.5. Soluble-Blys binding assay

The 96-well plate coated with Blys (1 μg/ml) was incubated at 4 °C overnight. After blocking with 1% (w/v) BSA/PBS for one hour, the 100 μl antibody dilutions or hybridomas was added to each well, and incubated for one hour. Plates were washed using PBST for three times, and 100 μl of goat anti-human IgG or goat anti-mouse IgG antibodies (diluted at a 1:10000 ratio using 0.5% (w/v) BSA/PBS, Jackson Immuno research) were added for one hour. Plates were again washed three times with PBST and then incubated with TMB substrate (Sigma). Finally, the OD value at 450 nm was measured with sunrise instrument (TECAN).

2.6. Membrane-bound Blys binding assay

CELL-based ELISA was used to assess the capacity of anti-Blys mAbs to bind to membrane-bound Blys.The 293C18 cells (ATCC), transfected with cDNA encoding membrane-bound Blys were immobilized onto poly-L-lysine coated 96-well plates at a density of 1×10^5cells/well. After PBS washing and blocking with 1% (w/v) BSA/PBS, 100 μl of serial dilutions of antibodies in diluent buffer were added to each well. The plates were later incubated for one hour at room temperature, washed 3 times with PBST, and then dispensed with 100 μl of Eu-N1 labeled anti-human IgG (100 ng/ml in diluent buffer, Perkin Elmer) into individual wells. Finally, bound mAbs were detected *via* streptavidin-Delfia (PerkinElmer).

2.7. Affinity measurement

Affinity measurements were carried out using the standard operation principle of Biacore T200 instrument (GE). The goat anti-human IgG was immobilized on CM5 chip according to the technical manual of capture kit and after five cycles of start-up, each antibody (10 μg/ml) was injected at a flow rate of 10 μl/min, for 100 s from the start of the cycle. This was followed by injecting Blys (300 nM) at a flow rate of 30 μl/min, for 300 s and the dissociation phase was monitored for 600 s. The association and dissociation rates were finally analyzed using the Biacore T200 evaluation software.

2.8. Blys receptor binding inhibition assay

Blys receptors (1 μg/ml, Sino Biological Inc.) were coated to 96-well plate at 4 °C overnight. After blockingwith1% (w/v) BSA/PBS for one hour, plates were washed using PBST two times. Next, 50 μl of biotinylated Blys (5 ng/ml final concentration)and 50 μl of antibody dilutions were added to well consecutively, and incubated for one hour. Plates were washed using PBST three times, followed by addition of 100ul Streptavidin-HRP dilutions (Pierce) to each well for one hour. Plates were washed three times with PBST, and were developed with 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate (Sigma Aldrich). The optical density (OD)measurements were performed using a sunrise instrument (TECAN).

2.9. Blocking activity of anti-Blys antibody in mouse model

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee. To evaluate the neutralizing activity of the Blys antibody, BALB/C mice