Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 133–140

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Qualitative and quantitative studies on human B7.1-Fc fusion protein and the application in pharmacokinetic study in rhesus monkeys

Zhihang Chen[a,b], Ruorui Liu[a,b], Jinjing Che[a,b], Hongjiao Shi[a,b], Yunan Hou[a,b], Yunlong Liu[a,b], Lili Sun[a,b], Li Tan[a,b], Chengqi Shan[a,b], Peisheng Hu[c,*], Yuanguo Cheng[a,b,**]

[a] State Key Laboratory of Pathogen and Biosecurity, Beijing 100071, PR China
[b] Department of Pharmacy, Beijing Institute of Microbiology and Epidemiology, No. 20, Dongda Street, Fengtai District, Beijing 100071, PR China
[c] Department of Pathology, Keck School of Medicine, University of Southern California, 2011 Zonal Avenue, HMR 205, Los Angeles, CA 90033, USA

ARTICLE INFO

Article history:
Received 5 April 2010
Received in revised form 7 July 2010
Accepted 26 July 2010
Available online 7 August 2010

Keywords:
B7.1-Fc
Enzyme immunoassay
Pharmacokinetics
Surface plasmon resonance
Flow cytometry

ABSTRACT

A sensitive, accurate, and precise enzyme immunoassay (EIA) for the quantification of intact human B7.1-Fc in rhesus monkey serum was validated, and the characteristics of B7.1 and Fc moiety of fusion protein were identified by surface plasmon resonance (SPR) and flow-cytometric method, respectively. B7.1-Fc bound to CD28 and CTLA-4 with K_d values of 45.1 and 9.58 nM, respectively, which were very closed to the previous reports and the function of Fc moiety of fusion protein was also confirmed by Fc receptor binding assay and IL-8 releasing assay. To monitor the intact protein, the EIA method employed a sandwich scheme in which a multiclonal anti-human IgG (Fc specific) antibody and a monoclonal anti-human B7.1 antibody were served as capture and detection antibody, respectively. This EIA has a range of reliable response of 0.5–32 ng/ml. The LLOQ was established at 0.5 ng/ml. The intra-assay precision and accuracy were 6.1–8.8% and (3.0–9.0)%, respectively with the inter-assay precision and accuracy were 5.7–11.5% and (10.7–9.1)%, respectively. Stability was established under certain conditions and no significant differences were found. This validated EIA assay was then successfully employed in the assessment of pharmacokinetic behavior of B7.1-Fc in rhesus monkeys after intravenous infusion, and a non-linear characteristics was established across the investigated dosage range (32–320 μg/kg).

1. Introduction

T lymphocytes are educated in the thymus to gain immune competence. Mature T cells migrate into secondary lymphoid organs where they encounter Ags, expand, and differentiate into effector cells. The activated T cells are then dispatched to target tissues to mediate effector function. Activation of naive T cells requires at least two signals for the optimum induction of cytokine (e.g. IL-2) and clonal expansion [1–3]. Antigen specificity is provided by the first signal which involves the engagement of an antigenic peptide in association with a major histocompatibility complex (MHC) molecule on antigen presenting cells (APC) with the T cell receptor/CD3 complex on T cells. A critical second or costimulatory signal can be provided by the interaction of B7 expressed on APC (activated B-cells, macrophage, and dendritic cells) with its receptors, CD28 and CTLA4, on T cells [4–6].

The B7 family currently has seven members: B7.1, B7.2, ICOSL, PD-L1, PD-L2, B7-H3 and B7-H4. Among these ligands, B7.1 (CD80) and B7.2 (CD86) were investigated most widely [7]. They both bind to CD28 and CTLA4, but they differ in their binding affinity, structure, and temporal expression. Considerable research has been done on the CD28/B7 costimulatory pathway [8,9]. Different ways of manipulating this pathway could provide insights into the mechanism and treatment of opposing pathological states. Block this pathway could result in immunosuppression, with implications for the treatment of autoimmune diseases, organ transplantation, and graft versus host disease [10]. Activating the CD28/B7 pathway could be valuable for the immune system to recognize and eliminate tumors that evade the immune system [11,12].

In the past few years, soluble costimulator proteins fused to the Fc portion of antibody have been developed and tested in mouse tumor models, such as murine B7.2-Ig [13–17]. Sturmhoefel et al. [13] and Swiniarski et al. [15] have reported that B7.2-Ig therapy as an adjuvant in cancer vaccines could produce complete tumor rejection in some tumor models. A study by Zhou et al. [18] showed that i.m. coinjection of B7.1/IgG1 Fc and carcinoembryonic antigen DNA plasmid can protect mice from the same tumor challenge.

* Corresponding author.
** Corresponding author at: Department of Pharmacy, Beijing Institute of Microbiology and Epidemiology, No. 20, Dongda Street, Fengtai District, Beijing 100071, PR China. Tel.: +86 10 66948493; fax: +86 10 66948547.
E-mail address: chenzhihang@nic.bmi.ac.cn (Y. Cheng).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.07.040

In our study described before [19], a B7.1 fusion protein consisting of the extracellular domains of human B7.1 and the Fc portion of human IgG1, called B7.1-Fc, was generated and evaluated for its antitumor potential when used alone or in combination with regulatory T (Treg) cell depletion. The similar tumor regression results were achieved even in the poorly immunogenic Madison 109 tumor. Rechallenge experiments done with mice that had sustained complete tumor regressions showed that these mice had immunological memory by their ability to reject subsequent implants.

Fusion with Fc portion of antibody was a strategy in common use to improve stability, increase solubility and prolong half-life of biotech drug. In addition, Fc portion also possessed many immunological functions, for example, opsonization, complement activation and ADCC (antibody-dependent cell-mediated cytotoxicity) which were almost all mediated by monocyte-macrophage system, because there existed many Fc receptors on the surface of those cells. As immunoregulation reagent, these functions of Fc portion in B7.1-Fc may be more noteworthy. Theoretically B7.1-Fc may act as a bifunctional molecule. Except for the key costimulation activity of B7.1 moiety, it could also induce and activate monocyte-macrophage system through the binding of Fc moiety with its receptor, and then play a synergistic role in tumor immunotherapy. Therefore, the costimulation ability of B7.1 moiety and the immune cell-activating ability of Fc moiety would be both investigated here.

The objective of the present study should be divided into two parts. Firstly, we qualitatively evaluated the characteristics of B7.1-Fc fusion protein to identify its mechanism and pathway of action *in vitro*. The activities of the two functional moieties, B7.1 and Fc, were studied by surface plasmon resonance (SPR) [20–22] and flow-cytometric method [23,24], respectively. Secondly, to quantitate intact B7.1-Fc in monkey serum, a new enzyme immunoassay (EIA) was developed and proper validated which was then successfully applied in delineating the pharmacokinetics (PK) in rhesus monkey after receiving a single intravenous bolus dose of B7.1-Fc. There were no reports in literature on the preclinical pharmacokinetic data up to now and our study may provide more information for the clinical study maybe carried out in the coming future.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

The murine/human chimeric monoclonal, Rituximab (Mabthera^{TM}) directed against the cell surface CD20 antigen in B cell lymphocytes was provided by Roche (Basel, Switzerland). INF-γ, human/mouse B7.2-Fc, mouse B7.1-Fc, human CTLA-4 Fc, CD28 Fc chimera and streptavidin-HRP were purchased from R&D Systems (Minneapolis, USA). Fetal calf serum (FCS) and RPMI 1640 were purchased from GIBCO (New York, USA). Amine coupling kit, CM5 sensor chip and Surfactant P-20 were purchased from BIAcore AB (Uppsala, Sweden). Pooled normal monkey sera were provided by Laboratory Animal Center of the Academy of Military Medical Sciences. Other materials were purchased from local suppliers at high purity.

2.1.2. Antibodies

Mouse monoclonal antibody against human CD64 (10.1), CD32 (FLI8.26), CD16 (3G8) and their isotype control were purchased from BD Pharmingen (San Diego, USA); anti-human B7.1 monoclonal antibody and its conjugate with biotin were purchased form R&D Systems (Minneapolis, USA). Anti-human IgG (Fc specific) multiclonal antibody and its conjugate with HRP were purchased from Bethyl (Montgomery, USA). FITC-conjugated $F(ab)_2$ fragment of goat anti-mouse IgG (Fab specific) and that of goat anti-human IgG (γ-chain specific) were purchased from Sigma–Aldrich (St. Louis, USA); Human IL-8 ELISA Kit was purchased from R&D Systems (Minneapolis, USA).

2.1.3. Cell lines

The human monocytic cell line THP-1 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences and maintained in RPMI 1640 medium supplemented with 10% FCS.

2.2. Apparatus

SDS-PAGE was carried out on Bio-Rad Miniprotein-3 cell system (Hercules, USA). Protein purity was identified using Agilent 1200 series HPLC system (Santa Clara, USA). Surface Plasmon resonance measurements were performed on a BIAcore 3000 instrument (Uppsala, Sweden). The cells were analyzed on the flow cytometry of BD FACSCalibur (San Diego, USA). Assay plates were washed with a Bio-Rad ImmunoWash Model 1575 (Schiltigheim, France) and measured on a Bio-Rad microplate reader Model 550 (Hercules, USA) with absorbance and reference wavelengths at 450 and 570 nm, respectively.

2.3. Animals

Rhesus monkeys were supplied by the Animal Raising Center of the Academy of Military Medical Sciences. The animals were individually housed in stainless-steel cages in a room with controlled temperature ($25 \pm 1\,^{\circ}C$) and humidity ($55 \pm 5\%$) and a 12-h light/dark cycle. The animals were fed with standard diet and had free access to water. All procedures involving animals and their care were carried out according to the guidelines of the Institutional Ethical Committee for Care and Use of Laboratory Animal of Academy of Military Medical Sciences in accordance with the governmental guidelines on animal experimentation, National Institutes of Health "Principles of Laboratory Animal Care".

2.4. Expression and purification of B7.1-Fc

Expression vector pEE12/B7.1-Fc encoding the human B7.1 signal and extracellular domains fused to the Fc region of human IgG1 was constructed, and the B7.1-Fc fusion protein was expressed in NS0 murine myeloma cells for long-term stable expression as described previously [19]. In brief, the highest producing clone was scaled up for incubation and the fusion protein was then purified by sequential protein A affinity chromatography and ion exchange chromatography. The fusion protein was then analyzed by SDS-PAGE and HPLC to show proper assembly and purity.

2.5. Avidity measurement of B7.1 moiety of B7.1-Fc

Binding experiments were performed by SPR on a BIAcore 3000 biosensor instrument. All experiments were performed at $25\,^{\circ}C$ using HBS buffer (10 mM HEPES with 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P-20; pH 7.4). Firstly, CTLA4-Ig and CD28-Ig were covalently coupled by primary amine groups to the carboxymethylated dextran matrix on a research grade CM5 sensor chip using the Amine Coupling Kit as follows: HBS was used as a mobile phase at a flow rate of 5 μl/min. The carboxymethylated dextran matrix of the sensor chip surface was first activated with an injection of 200 μl of the NHS/EDC reagent mixture, then CTLA4-Ig (10 μg/ml in 10 mM sodium acetate; optimal pH 4.5) or CD28-Ig (15 μg/ml in 10 mM sodium acetate; optimal pH 5) were injected for 4 min. After coupling, the unreacted sites were blocked by injection of 35 μl of ethanolamine (pH 8.5; 1 M).

During analysis, B7.1-Fc solution with different concentrations (1 mM and four twofold dilutions thereof) in duplicate was injected separately and passed over the sensor surface with HBS at a flow rate of 20 μl/min. This was followed by a HBS buffer wash. Finally, a regeneration step was performed by injecting 10 μl of 10 mM glycine–HCl (pH 2.5) after each analysis cycle. To correct for nonspecific binding and bulk refractive index changes, sensor response to a blank channel run simultaneously without immobilized receptor protein on the sensor chip surface was subtracted for each experiment. Sensorgrams for all binding interactions were recorded in real time and were analyzed using BIAevalution software version 4.0. The 1:1 Langmuir binding model was chosen to global fit the data by choosing fit kinetics simultaneous k_a/k_d with χ^2 value as a standard statistical measure of the closeness of fit.

2.6. Immune cell-activating ability of the Fc moiety of B7.1-Fc

2.6.1. Characterization of the Fc receptor population on THP-1 cells

The characteristics of the receptors on THP-1 were investigated using three monoclonal antibodies: mouse monoclonal antibody against CD64 (clone 10.1), CD32 (clone FLI8.26) and CD16 (clone 3G8). THP-1 cells, washed in PBS, at 5×10^5 cells/tube were incubated with each of the mouse monoclonal antibodies or their isotype controls for 45 min at 4 °C. The cells were then washed in ice-cold saline and incubated with 50 μl of FITC-conjugated $F(ab)_2$ fragment of goat anti-mouse IgG antibody (1:20 dilution) for another 30 min. After a wash in saline, the cells were resuspended in 0.5 ml saline and analyzed on the flow cytometry FACSCalibur. Using isotype control tube to adjust the FSC (Forward Scatter)/SSC (Side Scatter) amplifier gain and voltage so that the population of interest is clearly visible towards the center of FSC/SSC dot plot, then adjust the threshold on FSC to eliminate the cell debris. Set a gate around the population of interest and adjust the voltage on fluorescence parameter of FL1 channel (for FITC) so that the autofluorescence falls in the middle of the first log. The instrument setting was then used to acquire data of the tested samples, and histogram plots and statistic parameters of staining positive cells were further analyzed by Cellquest software.

2.6.2. Fc receptor binding assay

THP-1 cells, washed in PBS, at 5×10^5 cells/tube were incubated with 100 μl of B7.1-Fc or Rituximab (as positive control) of different concentrations, at 4 °C for 45 min. After washing with cold saline, the cells were incubated with 50 μl of FITC-conjugated $F(ab)_2$ fragment of goat anti-human IgG (γ-chain specific) (1:20 dilution of 1.0 mg/ml solution) at 4 °C for a further 30 min. The cells were washed, again in cold saline, and suspended in 0.5 ml PBS and analyzed by flow cytometry. The sample untreated with B7.1-Fc or Rituximab was served as negative control.

The effect of protein was studied by the addition of ultra pure human albumin to PBS, used as FITC-conjugated detection antibody diluents.

2.6.3. IL-8 releasing assay

B7.1-Fc or Rituximab (as positive control) were serially diluted with D-PBS, and immobilized onto a 96-well plate (100 μl/well, 4 °C; overnight). The IFN-γ-activated THP-1 cells were prepared by culturing for 48 h in the presence of 100 U/ml (10 ng/ml) IFN-γ. After culturing with INF-γ, the THP-1 cells were washed with RPMI 1640 medium containing 10% FCS, and adjusted to 3×10^6 cells/ml with the same medium, which was then plated on a B7.1-Fc-immobilized 96-well plate at 100 μl/well, and cultured for about 18 h. The cells after culturing were centrifuged (1500 rpm, 15 min), the culture supernatant was recovered at aliquots of 50 μl, and the amount of IL-8 in the supernatant was measured by the human IL-8 ELISA kit. ELISA measurement was performed in triplicate.

As a control, the THP-1 cells not activated with IFN-γ were similarly plated on wells on which 100 μg/ml of B7.1-Fc or Rituximab had been immobilized, and the amount of IL-8 produced was measured.

2.7. Development of EIA for quantitation of intact B7.1-Fc

2.7.1. Optimization of the antibodies

To set up an ideal EIA method for detection of intact B7.1-Fc, a sandwich Enzyme-linked immunosorbent assays (ELISA) scheme was adopted in which capture and detection antibodies could recognize B7.1 and Fc moieties of the fusion protein, respectively, or in reverse order. Two mouse anti-human B7.1 monoclonal antibodies (one conjugated with biotin) and two goat anti-human IgG (Fc specific) multiclonal antibodies (one conjugated with HRP) were selected and combined into two antibody pairs. To eliminate the cross-reaction of anti-human IgG (Fc specific) multiclonal antibody with endogenous IgG protein in monkey serum, the multiclonal antibody was firstly cross-absorbed by the immunoaffinity columns immobilized monkey IgG to remove the components cross-reacted with monkey IgG.

The optimum dilutions for the above antibodies were predetermined through checker-board titrations. The procedures were carried out in the same way as blow (see Section 2.7.2) except followings: A microtiter plate was coated with 100 μl of anti-human IgG_{Fc} antibody (capture Ab) at different concentrations (0.5, 1, 5 and 10 μg/ml) in carbonate buffered saline CBS (50 mM, pH 9.6) overnight at 4 °C. After blocking, the wells were then incubated with 100 μl of positive sample (containing B7.1-Fc at 10 ng/ml) and negative sample (0 ng/ml of B7.1-Fc) for 1 h, followed by addition of 100 μ1 of biotin-conjugated mouse anti-human B7.1 antibody (detection Ab) at different dilutions (1:50, 1:100, 1:200 and 1:400).

The concentrations of mouse anti-human B7.1 antibody (capture Ab) and HRP conjugated anti-human IgG_{Fc} antibody (detection Ab) were optimized using similar method, but the step of signal amplification using biotin/streptavidin system was not needed.

2.7.2. EIA procedure

Calibration standards were prepared over the range 0.5–32 ng/ml B7.1-Fc in pooled monkey serum. A zero serum calibration standard and serum quality control (QC) samples at three levels (1, 4 and 16 ng/ml) were also analyzed. These represent QC concentrations were in the lower quartile, between the second and third quartiles, and in the upper quartile of the standard curve range, respectively.

A clear polyvinylchloride (PVC) microtiter plate was coated with 100 μl of anti-human IgG (Fc specific) antibody (5 μg/ml in CBS) overnight at 4 °C. For the remainder of the assay, the plate was incubated at 25 °C. The antibody solution was aspirated and the remaining sites were blocked by the addition of 300 μl of 5% skim milk in PBS (pH 7.4) for 2 h. This was followed by five 300 μl washes with PBS containing 0.05% Tween 20 (PBST). Then, 100 μ1 of the standards, QC samples, and study samples were added to the plate. Following a 1 h incubation the plate was washed with PBST as before and 100 μ1 of biotin-conjugated mouse anti-human B7.1 antibody (1:200 diluted) was added and incubated for 1 h. After a wash with PBST, 100 μ1 of 1:200 diluted streptavidin–HRP was added and incubated for 20 min, and then 100 μl of TMB peroxidase substrate solution was added and incubated for another 20 min. Lastly, the color development was stopped by the addition of 50 μ1 of 2 M H_2SO_4 and the absorbance of each well was determined by the microtiter plate reader.

2.7.3. Validation procedure

The EIA was validated in accordance with FDA guidelines for Bioanalytical Method Validation [25].

The specificity of the EIA is evaluated by running standards of several similar compounds of available species (including mouse B7.1-Fc, human B7.2-Fc, mouse B7.2-Fc, human CD28-Ig and CTLA4-Ig) in the assay to determine if any compounds could be detected individually by the assay. Furthermore, B7.1-Fc QC samples were assayed in the presence of up to a 1000-fold molar excess (5 μg/ml) of the above compound, respectively. The observed QC concentrations of B7.1-Fc in the presence and absence of similar compounds were compared.

Matrix effects were investigated by comparing the calibration curves in different biological matrix (including 100%, 50% and 20% pooled monkey serum) with that in D-PBS. To study the effect of dilution, serum samples with two high concentrations (0.1 and 5 μg/ml) were prepared and diluted (10-, 100-, 500- or 5000-fold) with certain matrix before assay in triplicate. Accuracy was determined by comparing observed with theoretical values.

A seven-point calibration curve ranging from 0.5 to 32 ng/ml of B7.1-Fc in 100% monkey serum was assayed in duplicate. The relationship between the normalized absorbance and B7.1-Fc concentration was described by a four-parameter logistic regression model of the form as below:

$$Y = \frac{a-d}{\left[1+(x/c)^b\right]} + d$$

where Y is the absorbance (A) and x is logarithmic concentration of B7.1-Fc. d and a represent the upper and lower asymptotes, respectively, of the curve and correspond to the theoretical absorbance of the assay at infinite and zero concentrations (A_{max} and A_{min}), respectively. c is the concentration associated with the point of symmetry of the sigmoid and is located at the midpoint of the assay found at the inflection point of the curve (EC_{50}). b is a curvature parameter and is related to the slope of the apparent linear region of the curve. The concentrations of the B7.1-Fc in the unknown samples were calculated according to the calibration curve constructed in the same run.

Sensitivity defined as lower limit of quantification (LLOQ) was evaluated by repeatedly assaying the lowest level standard with 0.5 ng/ml of B7.1-Fc in pooled monkey serum obtained from six different sources. Intra-assay accuracy and precision were calculated from a single run of six replicates of QC samples with concentrations of 1, 4 and 16 ng/ml of B7.1-Fc in serum, and inter-assay performance calculated from six separate runs of five replicates with same concentrations. Estimates of precision were expressed as the coefficient of variation (CV) relative to the overall mean observed concentration at each concentration level. Estimates of accuracy were expressed as the percentage of the overall mean observed concentrations versus the corresponding theoretical concentration.

Stability of B7.1-Fc prepared in monkey serum was assessed with five aliquots at each of the low and high concentrations (1 and 16 ng/ml, respectively). Long-term storage stability was tested at −70 °C for 2 months. Short-term storage stability was evaluated at 4 °C and room temperature for 24 h. Freeze–thaw stability was assessed over three repetitive freeze (−70 °C) and thaw (4 °C) cycles.

2.8. Pharmacokinetic application

2.8.1. Experimental design and dosage groups

The developed method was used to evaluate the pharmacokinetics of B7.1-Fc in rhesus monkeys. Nine male rhesus monkeys (weighing 4.5 ± 0.3 kg) were used in pharmacokinetics experiments. Monkeys were divided into three groups by a simple randomization method. A 30-min intravenous infusion of 32, 100, or 320 μg/kg dose was administered to study the linear characteristics of pharmacokinetics. The blood samples were drawn from the femoral veins of the animals using a puncture needle before dosing (0 h), at 15, 30 min during intravenous infusion and 5, 30 min, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h after dosing. Serum samples were collected and kept at −20 °C before analysis.

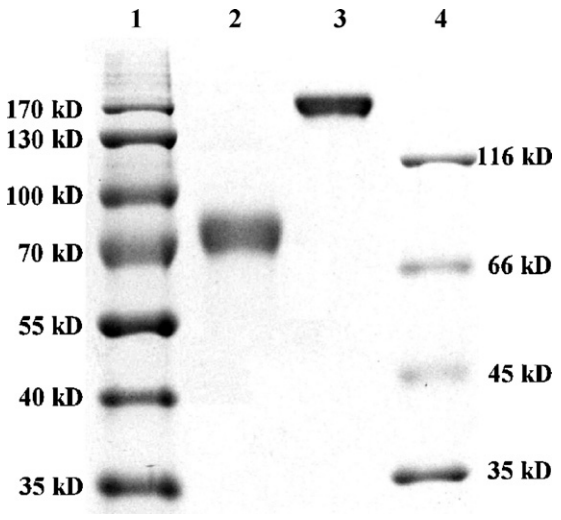

Fig. 1. Electrophoretic analysis of B7.1-Fc by 10–20% Tris–glycine SDS-PAGE (stained by Coomassie blue). Extracellular domains of human B7.1were fused to the NH2 terminus of the Fc portion, including hinge, CH2, and CH3 of human IgG1 by PCR to produce a dimeric protein. (1) and (4) molecular weight markers; (2) reduced B7.1-Fc; (3) non-reduced B7.1-Fc.

2.9. Statistic analysis

Pharmacokinetic data analysis was performed by the noncompartmental method using Pharsight® WinNonline 5.2.1 (St. Louis, USA). All parameters are expressed as mean ± S.D. Dose proportionality after a single intravenous infusion administration of different dosages was evaluated by comparison of the dose normalized C_{max} and $AUC_{0-\infty}$ across dosage levels using an ANOVA and linear regression analysis. A P-value below 0.05 indicates significant difference between data means.

3. Results

3.1. Expression and purification of B7.1-Fc

The theoretical molecular weight of dimeric B7.1-Fc was about 160 kD which was identified by SDS-PAGE analysis shown in Fig. 1 and its dimeric nature was also confirmed. The purity was proved to be greater than 95.0% by high-performance liquid chromatography (HPLC).

3.2. Receptor binding ability of B7.1 moiety

Interactions of B7.1-Fc with its receptors, CD28 and CTLA4, were measured by SPR biosensor. The pH value of sodium acetate buffer used for immobilization was firstly optimized (Fig. 2A) and BIAcore response of about 5000 RU (resonance unit) was achieved after immobilization. B7.1-Fc (0.25 mM) bound to a very similar level when injected at the beginning and end of the experiment (data not shown), indicating that the immobilized receptors were stable. The experimental signals were shown in Fig. 2B with the fits calculated with 1:1 Langmuir binding model. The kinetic parameters obtained were shown in Table 1. It was clear that the B7.1 moiety

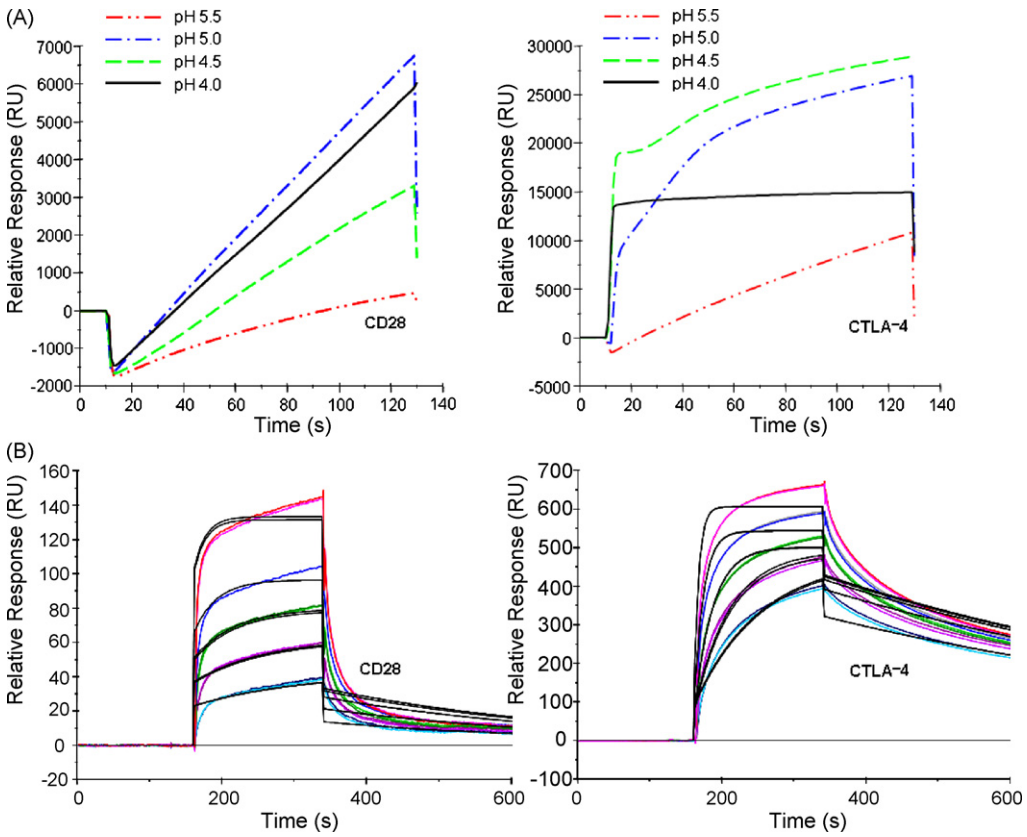

Fig. 2. Measuring the affinity of B7.1-Fc binding to its receptors, CD28 and CTLA-4, by a Surface Plasmon Resonance assay. (A) The optimization of pH value of sodium acetate buffer when CD28 and CTLA-4 immobilized on CM5 sensor chip. (B) Kinetics of the interactions of B7.1-Fc with its receptors. CD 28 and CTLA-4 were bound to CM5 chips, respectively, and a range of B7.1-Fc concentrations (1 mM and four twofold dilutions thereof) were injected sequentially for a 3-min contact time at 20 μl/min through a flow cell with CD28 (5322 RUs), CTLA-4(4982 RUs) or no protein (control) immobilized. The experimental signals were shown with the fits calculated with 1:1 Langmuir binding model.

of fusion protein can specifically bind to its receptors, CD28 and CTLA-4. A K_d value of 45.1 nM was obtained for CD 28 with a binding maximum of 34.8 RUs, and a K_d of 9.58 nM was achieved for CTLA-4 with a binding maximum of 434 RUs.

3.3. Immune cell-activating ability of Fc moiety

The ability of antibodies against CD64, CD32 and CD16 to bind to the cell line is shown in Fig. 3. Only anti-CD64 and anti-CD32 antibodies bound to the cell line showing the presence of the FcγRI and FcγRII receptors and the absence of FcγRIII receptors. This agrees with literature reports that FcγRIII receptors are expressed only in a subpopulation of monocytes [26].

The effect of human albumin at concentrations of 0.1–0.5% on Fc receptor binding assay was investigated and there were no significant differences observed (data not shown). Binding studies were therefore carried out at the intermediate concentration of 0.2% human albumin. In Fig. 4A, the Fc receptor binding curve of B7.1-Fc was compared to the positive control (Rituximab). There were significant differences between the dose–response curve of test B7.1-Fc and that of control. The receptor binding ability of Fc moiety of fusion protein was nearly decreased by one-half compared with whole antibody molecular.

Furthermore, the activity of immune cell-activating of Fc moiety was quantified by monitoring the producing of IL-8 from THP-1 cell by stimulation via Fc receptor. As a result, as shown in Fig. 4B, the effect of promoting IL-8 production by B7.1-Fc was identified, and the amount of IL-8 produced by the THP-1 cells increased depending on the amount of immobilized B7.1-Fc. When B7.1-Fc was not

Table 1
Kinetic parameters of binding interactions between B7.1-Fc and its receptors, CD28 and CTLA-4.

	k_a ($\times 10^5$ M^{-1} s^{-1})	k_d ($\times 10^{-3}$ s^{-1})	K_a ($\times 10^7$ M^{-1})	K_d ($\times 10^{-9}$ M)
CD28	0.59	2.67	2.22	45.1
CTLA-4	1.51	1.45	10.4	9.58

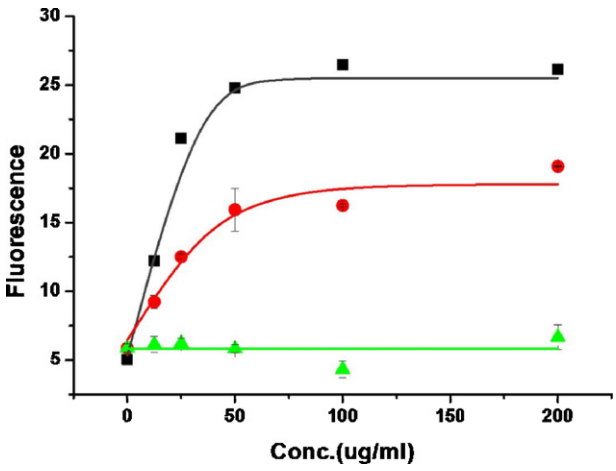

Fig. 3. Characterization of THP-1 cell surface receptors by flow cytometry using mouse anti-human FcγR receptor antibodies and FITC-conjugated secondary antibody ($F(ab)_2$ fragment of goat anti-mouse IgG antibody). ■ = anti-CD32; ● = anti-CD64; ▲ = anti-CD16. Data were presented as mean ± S.D. (n = 3).

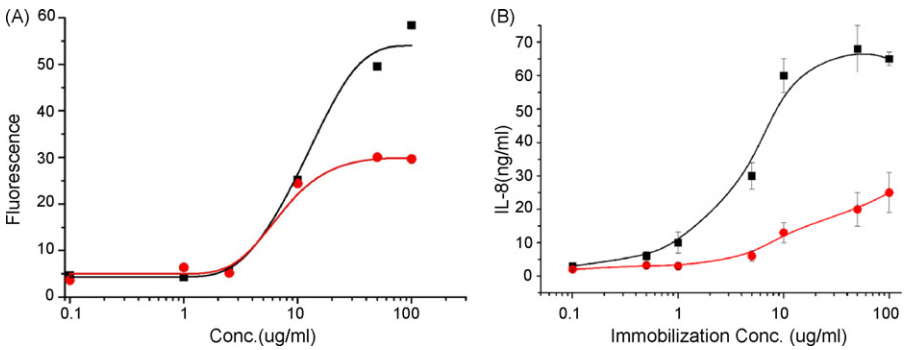

Fig. 4. Comparison of the dose–response curves for B7.1-Fc and positive control (Rituximab). (A) Receptor binding assay. THP-1 cells were incubated with different concentrations of B7.1-Fc or Rituximab and then determined using FITC-conjugated $F(ab)_2$ fragment of goat anti-human IgG by flow cytometry. (B) IL-8 releasing assay. THP-1 cells activated by IFN-γ were incubated in a 96-well plate immobilized different concentrations of B7.1-Fc or Rituximab, then the amount of IL-8 in culture supernatant was detected by ELISA Kit. ■ = Rituximab; ● = B7.1-Fc. Data were presented as mean ± S.D. (n = 3).

immobilized and when the THP-1 cells were not activated with IFN-γ, very little production of IL-8 by the THP-1 cell could be detected. The ability of B7.1-Fc in inducing IL-8 production was significantly less than Rituximab which was consistent with the above obtained by flow cytometry.

3.4. Validation of EIA for determination of intact B7.1-Fc

Comparing two ways to measure the intact B7.1-Fc, it was proved that the sequence of anti-human IgG_{Fc} capture and then anti-human B7.1 detection was superior to that of anti-human B7.1 capture and then anti-human IgG_{Fc} detection. The sensitivity was increased to at least five times as compared with the latter (data not shown).

According to the checker-board titration results, the concentration of capture antibody (anti-human IgG_{Fc}) was determined to be 5 μg/ml with the detection antibody (anti-human B7.1) at a dilution of 1:200, which was used in the following validation.

3.4.1. Specification

The assay exhibited no detectable cross-reaction with mouse B7.1/Fc, mouse B7.2/Fc, human B7.2/Fc, human CD28/Fc and CTLA-4/Fc when these analogs were determined individually using our EIA method.

When B7.1-Fc QC samples were assayed in the presence of 5 μg/ml of the above compound, the assay values by the EIA were mainly from B7.1-Fc because the observed values were much closed to those in absence of analog (data not shown). This result indicated that the addition of these analogs did not affect the binding of B7.1-Fc to its antibodies and the assay has good specificity for B7.1-Fc determination.

3.4.2. Matrix effects and dilution effects

The calibration curves were prepared in different matrix including D-PBS, 20%, 50% and 100% pooled monkey serum, as shown in Fig. 5A. Compared with PBS, the biologic matrix, to different extent, exhibited matrix suppression mainly resulted from the interference of the IgG in monkey serum. To keep consistent and eliminate variance, 100% pooled serum was used as diluents throughout the following assay.

Results obtained from serum samples diluted 10-, 100-, 500- or 5000-fold with 100% pooled monkey serum prior to analysis showed that accuracy was from −12.2% to 10.8%, indicating the validity of determination of B7.1-Fc in over-range samples.

3.4.3. Calibration curve fitting and sensitivity

A series of dilutions from 0.5 to 32 ng/ml of B7.1-Fc in monkey serum was prepared and assayed. A four-parameter logistic model was used to fit the standard curve with a typical equation shown in Fig. 5B. Calibrators at each level, when back-calculated using the fitted curve could fall within ±15% of the theoretical value, which indicated the model fitted well to B7.1-Fc.

LLOQ was determined by six replicates of samples in pooled monkey serum at lowest level of B7.1-Fc (0.5 ng/m1) on three consequent runs. With the accuracy and precision below ±13.2% and 9.4%, respectively, 0.5 ng/ml was defined as LLOQ of the assay for determination of intact B7.1-Fc.

3.4.4. Accuracy and precision

Summary of the intra- and inter-assay precision and accuracy data for the determination of B7.1-Fc in monkey serum were presented in Table 2. For intra-assay, the coefficient values (CVs) of QC samples at concentrations of 1, 4 and 16 ng/ml were all below 8.8% and the accuracy of QC samples were between −3.0% and 9.0%. For inter-assay, the CV values of QC samples were less than 11.5% and

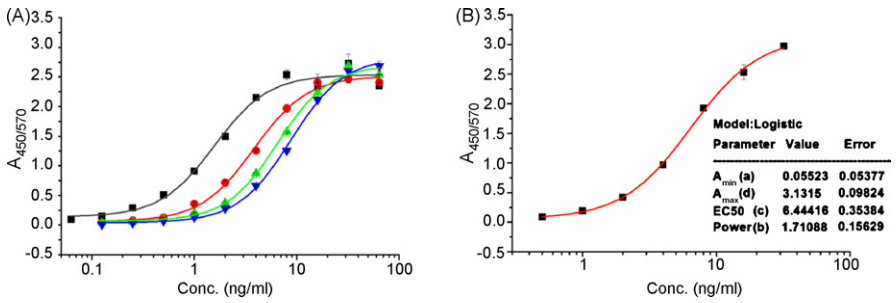

Fig. 5. (A) Matrix effects determined by comparing the calibration curves prepared in different matrix.■ = D-PBS; ● = 20% monkey serum; ▲ = 50% monkey serum; ▼ = 100% monkey serum in D-PBS. (B) The typical calibration curve of B7.1-Fc quantitated by EIA in 100% monkey serum. The fitted curve was based on the four-parameter Logistic model. Data were presented as mean ± S.D.

Table 2
Assay precision and accuracy for the determination of B7.1-Fc in rhesus monkeys.

	Theoretical concentration (ng/ml)	Observed concentration (ng/ml, mean ± S.D.)	Precision[a] (%)	Accuracy[b] (%)
Intra-assay				
Low QC	1	1.09 ± 0.07	6.4	9.0
Medium QC	4	3.88 ± 0.34	8.8	−3.0
High QC	16	17.11 ± 1.04	6.1	6.9
Inter-assay				
Low QC	1	0.89 ± 0.08	9.0	−10.7
Medium QC	4	4.36 ± 0.25	5.7	9.1
High QC	16	15.15 ± 1.74	11.5	−5.3

[a] Precision = (S.D./mean) × 100.
[b] Accuracy = [(mean observed concentration − theoretical concentration)/theoretical concentration] × 100.

the accuracy of QC samples was from −10.7% to 9.1%. These results confirm that the assay has good intra-/inter-assay precision and accuracy.

3.4.5. Stability

The storage stability of B7.1-Fc in serum matrix was assessed at intended storage temperatures for different periods (2 months at −70 °C, 24 h at 4 °C and room temperature) and the influence of freeze–thaw cycles was studied at −70 °C for at least three cycles. The results showed the percentage difference from theoretical concentration and the coefficient of variation were similar to those of the inter-assay test and no significant differences were detected under these conditions.

3.5. Pharmacokinetics of B7.1-Fc in rhesus monkeys

Serum concentration–time profiles of B7.1-Fc in rhesus monkeys following intravenous infusion at 32, 100 and 320 μg/kg were shown in Fig. 6, and corresponding mean pharmacokinetic parameters were listed in Table 3. B7.1-Fc reached peak serum concentration rapidly following intravenous infusion administration with a T_{max} of 0.5 h for all three doses. Average C_{max} ranged from 0.17 ± 0.04 to 5.53 ± 0.59 μg/ml, and $AUC_{0-\infty}$ ranged from 1.55 ± 0.17 to 35.94 ± 8.39 μg h/ml. B7.1-Fc elimination followed a bi-exponential profile with a $t_{1/2}$ ranging from 22.02 ± 2.11 to 32.26 ± 3.84 h.

Based on the weighted regression analysis of the pooled data, C_{max} and $AUC_{0-\infty}$ values increased as the dose increased in a linear manner, but more than proportionally. When the dose of B7.1-Fc increased in a ratio of 1:3:10, the $AUC_{0-\infty}$ values increased in a ratio of 1:6:24 and the C_{max} values increased in a ratio of 1:8:32. This coincided with a decrease in total body clearance ($P < 0.001$) and elimination half-life ($P < 0.001$), consistent with non-linear disposition. Therefore, our results support non-linear rather than linear serum pharmacokinetics of B7.1-Fc across the investigated dosage range in monkeys (32–320 μg/kg).

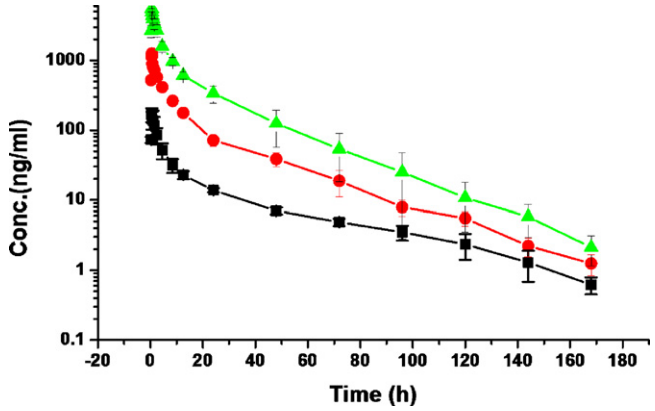

Fig. 6. The serum concentration–time profiles of B7.1-Fc in rhesus monkeys during and after intravenous infusion administration of different dosage. ■ = 32 μg/kg; ● = 100 μg/kg; ▲ = 320 μg/kg. Symbols represented the observed data (mean ± S.D.).

4. Discussion

As the major costimulator, B7 family plays a key role in the activation and differentiation of T cells and then induces some T cell effector functions in target tissues, including tumors. In the past few years, recombinant costimulator proteins have been widely investigated and exhibited some exciting application prospects. To provide more detailed information for preclinical and further clinical study, we identify the characteristics of B7.1-Fc fusion protein, constructed as before [19], by SPR and flow cytometry, and then develop a new sensitive EIA method to quantitate intact B7.1-Fc in monkey serum which applied successfully in pharmacokinetic study.

It was reported that B7.1 binds CD28 and CTLA-4 with affinities (K_d values ~200 and 12 nM, respectively) those were high when compared with other molecular interactions that contribute to T cell–APC recognition [27,28]. In the present study, we used surface plasmon resonance to measure the affinity and kinetics. At 25 °C, soluble B7.1-Fc bound to CD28 and CTLA-4 with K_d values of 45.1 and 9.58 nM, respectively, which were very closed to the previously reports (in the same order of magnitude of 10^{-9}).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a phenomenon which is exhibited by the antigen-binding ability of Fab moiety of an antibody and the immune cell-activating ability of Fc moiety of an antibody. To investigate whether the Fc moiety of our B7.1-Fc fusion protein could also exhibit the relative immune cell-activating ability, the Fc receptor binding curves of THP-1 cell

Table 3
Pharmacokinetic parameters following a single intravenous infusion of B7.1-Fc in rhesus monkeys ($n = 3$ per group).

Parameter[a,b]	Unit	Dosage of administration		
		32 μg/kg	100 μg/kg	320 μg/kg
AUC_{0-t}	μg h/ml	1.52 ± 0.16	9.08 ± 1.09	35.85 ± 8.40
$AUC_{0-\infty}$	μg h/ml	1.55 ± 0.17	9.14 ± 1.10	35.94 ± 8.39
MRT	h	30.66 ± 5.02	20.51 ± 2.38	16.86 ± 3.64
CL	ml/(kg h)	20.76 ± 2.43	11.05 ± 1.32	9.22 ± 2.05
V_{ss}	ml/kg	637.38 ± 126.05	225.43 ± 24.38	150.52 ± 2.54
$t_{1/2}$	h	32.26 ± 3.84	25.01 ± 1.44	22.02 ± 2.11
C_{max}	μg/ml	0.17 ± 0.04	1.31 ± 0.12	5.53 ± 0.59
T_{max}	h	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00

[a] AUC_{0-t}: area under the concentration–time curve from zero up to last quantifiable sample; $AUC_{0-\infty}$: area under the concentration–time curve from zero up to infinity; MRT: mean residence time; *CL*: total body clearance; V_{ss}: apparent volume of distribution at steady-state; $t_{1/2}$: half-life of elimination; C_{max}: maximum serum concentration; T_{max}: time to maximum concentration.
[b] The pharmacokinetic parameters were estimated from the data shown in Fig. 6. Data were presented as mean ± S.D.

expressed FcγRI and FcγRII receptors were plotted by flow cytometry and the changes in IL-8 production induced by the binding of the Fc moiety to the Fc receptor were monitored. The function of Fc moiety of B7.1-Fc was identified, but it was decreased significantly compared with control.

The main biologic functions of Fc portion of antibody include opsonization, complement activation, ADCC and transcytosis. However, the maintenance of these functions was depended on not only Fc portion, but also Fab portion of a whole antibody *via* certain signal cross-link. In the fusion protein B7.1-Fc, the Fab portion was displaced by B7.1 extracellular domain and the changes in the antigen-binding ability would inevitably influence the immune cell-activating ability of the Fc moiety, which was consistent with the fact observed in our study that the function of Fc moiety was nearly decreased by one-half compared with whole antibody molecule. Nevertheless, it may be still valuable for B7.1-Fc by targeted inducing of monocyte-macrophage system and playing a synergistic role in immunoregulation.

For determination of intact B7.1-Fc, a new EIA method was described and validated here. Considering the homology of species between monkey and human, the coated antibody (anti-human IgG_{Fc} antibody) has been pretreated to maximize eliminate the cross-reactive components. But monkey serum matrix still exhibited matrix suppression to some degree, therefore, the blank serum of monkey should be measured prior to pharmacokinetic study. Monkeys with low background of IgG were then selected for further administration and the serum samples should be diluted only using 100% pooled monkey serum during EIA analysis to keep consistence and increase accuracy. The specificity, sensitivity, precision, accuracy and stability of the assay were eventually confirmed to be all in the acceptable range.

The validated EIA method was then applied in pharmacokinetics studied in rhesus monkey which could describe the real changes of the whole B7.1-Fc molecular rather than the mixture of intact and metabolized B7.1. Following a single intravenous infusion of B7.1-Fc administration at 32, 100 and 320 μg/kg, a nonlinear pharmacokinetic property was established which have not been reported before.

Acknowledgement

This work was supported by a grant from Major New Drug Development of the National Science and Technology Major Projects of China's 11th 5-year Plan (No. 2009ZX09102-211).

References

[1] S.D. Norton, L. Zuckerman, K.B. Urdahl, et al., The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells, J. Immunol. 149 (1992) 1556–1561.
[2] F.A. Harding, J.P. Allison, CD28–B7 interactions allow the induction of $CD8^+$ cytotoxic T lymphocytes in the absence of exogenous help, J. Exp. Med. 177 (1993) 1791–1796.
[3] E.A. Harding, J.G. McArthur, J.A. Gross, et al., CD28-mediated signaling costimulates murine T cells and prevents induction of energy in T-cell clones, Nature 356 (1992) 607–609.
[4] D.J. Lenschow, T.L. Walunas, J.A. Bluestone, CD28/B7 system of T cell costimulation, Annu. Rev. Immunol. 14 (1996) 233–258.
[5] W.J. Metzler, J. Bajorath, W. Fenderson, et al., Solution structure of human CTLA-4 and delineation of a CD80/CD86 binding site conserved in CD28, Nat. Struct. Biol. 4 (1997) 527–531.
[6] R.J. Greenwald, G.J. Freeman, A.H. Sharpe, The B7 family revisited, Annu. Rev. Immunol. 23 (2005) 515–548.
[7] B.M. Carreno, M. Collins, The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses, Annu. Rev. Immunol. 20 (2002) 29–53.
[8] B.M. Carreno, L.L. Carter, M. Collins, Therapeutic opportunities in the B7/CD28 family of ligands and receptors, Curr. Opin. Pharmacol. 5 (2005) 424–430.
[9] A.H. Sharpe, A.K. Abbas, T-cell costimulation – biology, therapeutic potential, and challenges, New Engl. J. Med. 355 (2006) 973–975.
[10] B. Salomon, J.A. Bluestone, Complexities of CD28/B7:CTLA-4 costimulatory pathways in autoimmunity and transplantation, Annu. Rev. Immunol. 19 (2001) 225–252.
[11] G. Zheng, A. Chen, R.E. Sterner, et al., Induction of antitumor immunity via intratumoral tetra-costimulator protein transfer, Cancer Res. 61 (2001) 8127–8134.
[12] M. Moro, A.M. Gasparri, S. Pagano, et al., Induction of therapeutic T-cell immunity by tumor targeting with soluble recombinant B7-immunoglobulin costimulatory molecules, Cancer Res. 59 (1999) 2650–2656.
[13] K. Sturmhoefel, K. Lee, G.S. Gray, et al., Potent activity of soluble B7-IgG fusion proteins in therapy of established tumors and as vaccine adjuvant, Cancer Res. 59 (1999) 4964–4972.
[14] X. Huang, M.K. Wong, H. Yi, et al., Combined therapy of local and metastatic 4T1 breast tumor in mice using SU6668, and inhibitor of angiogenic receptor tyrosine kinases, and the immunostimulator B7.2-IgG fusion proteins, Cancer Res. 62 (2002) 5727–5735.
[15] H Swiniarski, K.S. Sturmhoefel, K. Lee, et al., Immune response enhancement by in vivo administration of B7.2Ig, a soluble costimulatory protein, Clin. Immunol. 92 (1999) 235–245.
[16] H. Zhou, M. Sequeira, M.E. Goad, et al., Efficacy and mechanisms of actions of rmB7.2-Ig as an antitumor agent in combination with Adriamycin and Cytoxan chemotherapy, Clin. Immunol. 101 (2001) 303–314.
[17] N. Yamaguchi, S. Hiraoka, T. Mukai, et al., Induction of tumor regression by administration of B7-Ig fusion proteins: mediation by type 2 $CD8^+$ T cells and dependence on IL-4 production, J. Immunol. 172 (2004) 1347–1354.
[18] Z.F. Zhou, Y. Peretz, Y. Chang, et al., Intramuscular gene transfer of soluble B7.1/IgG1 fusion cDNA induces potent antitumor immunity as an adjuvant for DNA vaccination, Cancer Gene Ther. 10 (2003) 491–499.
[19] A.H. Liu, P.S. Hu, L.A. Khawli, et al., Combination B7-Fc fusion protein treatment and Treg cell depletion therapy, Clin. Cancer Res. 11 (2005) 8492–8502.
[20] S. Hashimoto, in: K. Nagata, H. Handa (Eds.), Principles of BIAcore, Springer Verlag, Tokyo, 2000, pp. 25–26.
[21] P. Skottrup, H. Stephen, F. Hanne, et al., Detection of fungal spores using a generic surface Plasmon resonance immunoassay, Biosens. Bioelectron. 22 (2007) 2724–2729.
[22] P.A. Merwe, D.L. Bodian, S. Daenke, et al., CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics, J. Exp. Med. 185 (1997) 393–403.
[23] I. Ramasamy, E. Tran, A. Charnock, et al., Flow-cytometric method for the quantitation of the Fc function of intravenous immunoglobulin preparations, Vox Sang. 78 (2000) 185–193.
[24] C.B. Marsh, J.E. Gadek, G.C. Kindt, et al., Monocyte Fcγ receptor cross-linking induces IL-8 production, J. Immunol. 155 (1995) 3161–3167.
[25] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM070107.pdf, 2001.
[26] J.E. Gessner, H. Heiken, A. Tamm, et al., The IgG Fc receptor family, Ann. Hematol. 76 (1998) 231–248.
[27] P.S. Linsley, W. Brady, M. Urnes, et al., CTLA-4 is a second receptor for the B cell activation antigen B7, J. Exp. Med. 174 (1991) 561–569.
[28] P.S. Linsley, W. Brady, L. Grosmaire, et al., Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation, J. Exp. Med. 173 (1991) 721–730.