Phase I trial of a humanized, Fc receptor nonbinding anti-CD3 antibody, hu12F6mu in patients receiving renal allografts

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Key words: CD3, humanized antibody, pharmacokinetics, enzyme immunoassay, first dose reaction

Abbreviations: ANOVA, analysis of variance; AUC, area under the concentration-time curve; CHO, Chinese hamster ovary; Cl, clearance rates; Cmax, maximum serum concentration; CRS, cytokine release syndrome; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; FcRn, neonatal Fc receptor; FITC, fluorescein isothiocyanate; GCP, good clinical practice; HAMA, human anti-mouse antibody; HLA, human leukocyte antigen; HRP, horseradish peroxidase; Keq, elimination rate constant; LOQ, limit of quantification; mAb, monoclonal antibody; MRT, mean retention time; PD, pharmacodynamics; PE, phycoerythrin; PK, pharmacokinetics; QC, quality control; RES, reticuloendothelial system; SAE, serious adverse event; SD, standard deviation; T1/2keq, terminal half-life; Tmax, time to reach peak concentration; Tmin, time to reach the minimal concentration; Vss, distribution volume

Hu12F6mu is an Fc-mutated, humanized anti-CD3 antibody developed in our lab. The aim of this study was to assess single dose escalation pharmacokinetics (PK) and safety profile of hu12F6mu and to measure the effects of the antibody on levels of circulating T cells over time. Twenty-seven patients receiving renal allografts were randomized to receive hu12F6mu intravenously at a single-dose of 2.5, 5 or 10 mg. The concentration-time data obtained by a validated ELISA method were subjected to non-compartmental PK analysis by DAS 2.1 software. Subgroups of CD2+, CD3+, CD4+ and CD8+ lymphocytes were monitored periodically by flow cytometry. Our results showed that hu12F6mu exhibited linear PK over the dose range of 2.5–10 mg. A significant decline in the proportion of T cells was observed immediately after the infusion, followed by a progressive increase occurring over the ensuing days of therapy. A significant negative correlation was observed between serum concentration of hu12F6mu and CD3+ cell proportion. Intravenous infusion of hu12F6mu was well-tolerated in patients receiving renal allografts. These results suggest that hu12F6mu may have potential as a therapeutic agent, although further studies are needed.

Introduction

Muromonab-CD3 (Orthoclone OKT3; Ortho Biotech Products., Raritan, NJ, USA), is the first monoclonal antibody (mAb) for in vivo use in humans.1 It was a powerful immunosuppressive agent for the treatment or prevention of acute rejection episodes following kidney, heart or liver transplantation,2,3 but the product was removed from the market.6 Its clinical use was limited because administration was associated with a human anti-mouse antibody (HAMA) response and severe first dose reaction7-11 that caused rapid clearance of injected antibodies and reduced efficiency of the product.12-14 A systemic first dose reaction has been consistently observed with muromonab-CD3 therapy, consisting of fever, chills, rigors, tachycardia, tachypnea, diar rhrea, nausea, vomiting and, in several cases, pulmonary edema and even death.14-16 In recent years, the two major side effects were overcome by genetic engineering technology. First, antibody humanization significantly reduced the HAMA responses. Second, first-dose reactions, which resulted from T-cell activation and concomitant cytokine release, could be avoided by eliminating Fc receptor (FcR) binding activities.17 As a result, several forms of humanized, anti-CD3 mAb18,19 containing mutations in the upper CH2 region (from positions 234–237) have been constructed and shown to have reduced affinity for Fc receptor. These Fc-mutated anti-CD3 mAbs were significantly less mitogenic to T cells compared with the parental mAbs.
12F6 is a murine anti-human CD3 mAb\(^2\) that competes with muromonab-CD3 for binding to human T cells and possesses similar T-cell suppression and activation properties compared with muromonab-CD3.\(^3\) Hu12F6mu is an Fc-mutated, humanized version of 12F6 that displayed a similar antigen-binding affinity and specificity compared with 12F6, but with much weaker FcR binding activity.\(^4\) Further studies indicated that hu12F6mu was significantly less potent in T-cell activation, but retained potent immunosuppression, suggesting it may have utility as an immunosuppressive drug with less immunogenicity and toxicity than muromonab-CD3.\(^5\)

In the Phase 1 study of hu12F6mu reported here, safety and pharmacokinetic (PK) properties of the antibody were assessed in a single-dose escalation study conducted in patients who received renal allografts. Hu12F6mu was administered intravenously at a single-dose of 2.5, 5 or 10 mg and the effects of the drug on circulating T cell levels were assessed over time.

**Results**

**Demographics.** Twenty-seven patients were randomized to receive a single dose of hu12F6mu at one of three levels. All subjects completed the study. Demographic characteristics were similar between treatment groups (Table 1). The majority of the renal transplant recipients were male (74.1%); the mean age was 37.6 y. There were no major differences in the cause of end-stage renal disease, presence or absence of panel-reactive anti-HLA antibodies, number of HLA DR mismatches between donor and recipient, duration of cold ischemia for the graft, the recipients’ past or coexistent medical conditions (data not shown).

**Validity of the method for determination.** A specific ELISA method was developed to determine the serum concentration of hu12F6mu. Specificity tests indicated that human immunoglobulin (Ig), TNF receptor-Ig fusion protein, anti-CD25 mAb or 50% human serum did not interfere with hu12F6mu quantification. When added to blank serum at 5, 20 and 100 ng·mL\(^{-1}\), hu12F6mu recovery rates were 92.0% ± 1.9%, 100.9% ± 2.9% and 99.75% ± 2.1%, respectively. The intra- and inter-assay coefficient of variations (CV) was no more than 6.4% and 6.1%, respectively. The range of serum concentration quantification was 5–100 ng·mL\(^{-1}\). Limitation of quantification (LOQ) of the assay was demonstrated to be 5 ng·mL\(^{-1}\), as the serum sample was diluted to a minimal ratio of 1:5. Samples that returned values above the LOQ were diluted into the assay range with pooled normal human serum and tested repeatedly in the assay. The validity of the method demonstrated that the ELISA assay was reliable for the determination of serum hu12F6mu levels.

**Pharmacokinetics.** The hu12F6mu concentration-versus-time data during treatment for the three treatment groups were shown in Figure 1. The PK parameters were summarized in Table 2. Following infusion of a single dose of hu12F6mu at 2.5, 5 or 10 mg, hu12F6mu serum concentrations reached a maximum soon after the infusion and then declined slowly. The last measurable concentration in the 2.5 and 5 mg group was at approximately 672 and 840 h, respectively. Individual serum concentrations of hu12F6mu were quantifiable up to 1,176 h in the 10 mg dose group.

As dose levels increased at a ratio of 1:2:4, the mean maximum observed hu12F6mu concentrations (\(C_{\text{max}}\)) and mean area under the concentration-time curve from time zero to infinity (\(\text{AUC}_{\text{z-\infty}}\)) among the three groups increased at a ratio of 1:2.2:4.6 and 1:2.3:5.2, respectively. \(C_{\text{max}}/\text{dose}\) values of 220.76 ± 44.4 L\(^{-1}\), 243.07 ± 42.68 L\(^{-1}\) and 254.24 ± 12.69 L\(^{-1}\) were indistinguishable for the three dose groups, suggesting that \(C_{\text{max}}\) increased in a generally dose-proportional manner in the three groups. The value for \(\text{AUC}_{\text{0-\infty}}/\text{dose}\) at the 5 mg dose level was similar to 10 mg dose group (\(p = 0.85\) and \(p = 0.877, t\)-test), while greater than 2.5 mg dose group (\(p = 0.032\) and \(p = 0.022, t\)-test, respectively). The lower \(\text{AUC}_{\text{0-\infty}}\) estimates at the 2.5 mg dose may be due to serum concentrations of hu12F6mu that fall below the limit of quantification before the terminal elimination phase.

Mean terminal half-life (\(T_{\text{1/2,\text{ke}}}\)) ranged from 132.1 ± 10.8 to 144.1 ± 14.9 h with no significant difference among the three

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**Table 1.** Subject characteristics mean ± SD

|               | 2.5 mg (n = 9) | 5 mg (n = 9) | 10 mg (n = 9) | p     |
|---------------|---------------|--------------|--------------|-------|
| Age (years)   | 40.90 ± 12.65 | 33.33 ± 11.95 | 38.89 ± 6.79 | 0.3063 (K-W H test) |
| Gender        | 6 males, 3 females | 8 males, 1 females | 6 males, 3 females | 0.3660 (CMH test) |
| Height (cm)   | 166.50 ± 7.38 | 167.11 ± 5.97 | 166.67 ± 5.41 |       |
| Weight (kg)   | 59.18 ± 9.06  | 56.99 ± 8.09  | 57.70 ± 6.84  |       |

**Figure 1.** Concentration-time curves of hu12F6mu after a single intravenous infusion of 2.5 mg (■, n = 9), 5 mg (●, n = 9) and 10 mg (▲, n = 9) antibody in kidney transplantation recipients. Data are expressed as mean ± SD.

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\(^2\) Based on previous studies, muromonab-CD3 binds to human T cells with higher affinity and specificity compared with 12F6, but with much weaker FcR binding activity. \(^3\) Further studies indicated that hu12F6mu was significantly less potent in T-cell activation, but retained potent immunosuppression, suggesting it may have utility as an immunosuppressive drug with less immunogenicity and toxicity than muromonab-CD3. \(^5\)
in accordance with $T_{1/2ke}$ of hu12F6mu. A maximum reduction of lymphocyte subset (decrease to 3.2% from baseline) was observed in CD3+ cells after the administration of 10 mg of antibody. Three patients receiving 10 mg dose achieved a maximal decline to approximately zero immediately after administration. The maximum percentage decline in the proportion of CD4+ was 5.4% from baseline, while that was 16.5 and 18.6% respectively in CD2+ and CD8+ cells. The results indicated that hu12F6mu had a greater influence on the CD3+ cells and CD4+ cells compared with that on CD2+ and CD8+ cells. No patients reported occurrence of opportunistic infections during the 49 day follow-up period.

Correlation between serum hu12F6mu concentration and percentages of lymphocyte subsets. The concentration-effect plots between serum concentration of hu12F6mu and corresponding lymphocyte subsets proportion (%) were presented in Table 4, from which a significant negative correlation was shown. Previous in vitro studies demonstrated that 90% of TCR modulation could be achieved in the presence of hu12F6mu at a concentration of 1,000 ng·mL$^{-1}$. In this in vivo study, at 2.5 mg dose group, the maximal decline of CD3+ cells was only 79.4%. In patients who received 5 mg of hu12F6m, 90% decline of CD3+ cells occurred from 2–12 h with hu12F6mu concentration from 5.4% to 3.2% at 2.5 mg group, the value of clearance prolonged slightly and the difference was statistically significant ($p = 0.002 < 0.05$, Kruskal-Wallis H-test). Dose related differences in clearance may be due to the lack of quantifiable concentrations beyond 672 h in most of the patients receiving 2.5 mg. With regard to volume of distribution, individual $V_s$ values in all dose groups ranged from 6032.4 ± 811.3 to 6511.6 ± 760.6 mL indicating minimal distribution of hu12F6mu into the extravascular space.

Lymphocyte subset proportions. Flow cytometry was performed to measure circulating CD2+, CD3+, CD4+ and CD8+ cells after infusion of hu12F6mu because effects on the levels of these cells may indicate the potential to mediate depletion of T cells. Significant clearance of the T cells from the peripheral circulation was noted, followed by a progressive increase occurring over the ensuing days of therapy (Figs. 2–5). These flow cytometry assay results were consistent with the total blood lymphocyte counts obtained by classical clinical methods (Hematology Analyzer) (Fig. 6). The maximum percentage decline in the proportion of CD4+ was 5.4% from baseline, while that was 16.5 and 18.6% respectively in CD2+ and CD8+ cells. The results indicated that hu12F6mu had a greater influence on the CD3+ cells and CD4+ cells compared with that on CD2+ and CD8+ cells. No patients reported occurrence of opportunistic infections during the 49 day follow-up period.

**Table 2.** Pharmacokinetic parameters of hu12F6mu after a single intravenous infusion at a dose of 2.5, 5 and 10 mg (n = 9)

| Parameters | 2.5 mg | 5 mg | 10 mg | p       |
|-----------|--------|------|-------|---------|
| AUC$_{0-\infty}$ | 67175.4 ± 10738.5 | 158523.3 ± 14301.2* | 349490.3 ± 50912.3** | <0.001 (ANOVA) |
| AUC$_{0-\infty}$/dose | 26870.2 ± 4295.3 | 31704.7 ± 2860.2* | 34949.0 ± 5091.2** | 0.003 (ANOVA) |
| MRT | 166.1 ± 8.2 | 189.5 ± 14.6** | 224.8 ± 16.7** | <0.001 (ANOVA) |
| $T_{1/2ke}$ | 132.1 ± 10.8 | 132.6 ± 12.0 | 144.1 ± 14.9 | 0.208 (ANOVA) |
| $C_l/F$ | 38.3 ± 7.5 | 31.8 ± 2.7 | 29.1 ± 3.8** | 0.002 (K-W H test) |
| $V_{ss}$ | 6386.3 ± 1473.2 | 6032.4 ± 811.3 | 6511.6 ± 760.6 | 0.534 (ANOVA) |
| $k_e$ | 0.0053 ± 0.0004 | 0.0053 ± 0.0005 | 0.0049 ± 0.0005 | 0.240 (ANOVA) |
| $C_{max}$ | 550.4 ± 113.2 | 1215.3 ± 213.4** | 2542.4 ± 126.9** | <0.001 (ANOVA) |
| $C_{max}$/dose | 220.76 ± 44.4 | 243.07 ± 42.68 | 254.24 ± 12.69 | 0.192 (K-W H test) |

Data are expressed as mean ± SD. *p < 0.05, **p < 0.01 vs. 2.5 mg group (Student’s t-test), †p < 0.01 vs. 5 mg group (Student’s t-test).
Serum cytokine levels. IFNγ, TNFα and IL-6 serum levels were below the detection limit (<15.6 pg·mL⁻¹, <15.6 pg·mL⁻¹ and <3.12 pg·mL⁻¹, respectively) in patients who received three different level doses of hu12F6mu.

Safety. Hu12F6mu was well-tolerated in all patients who received the antibody. No serious adverse events (SAE) were observed in any treated patients after antibody infusion and no patient developed delayed graft function or acute rejection during therapy period. In addition, no serious laboratory events were observed during the study.

Discussion

Our previous study demonstrated that hu12F6mu had reduced T-cell activation potency, but maintained important immunosuppressive ability in vitro. In this Phase 1 study, our objective was to investigate the safety, PK and pharmacodynamic properties of hu12F6mu in patients receiving renal allografts. The hu12F6mu treatment was well-tolerated in all patients and no...
symptoms of cytokine release syndrome (CRS) were observed. No dose-limiting toxicity was observed after intravenous administration of doses in the range of 2.5–10 mg. A maximum tolerated dose was not established.

Our data suggest that hu12F6mu exhibited a proximately linear PK profile over the dose range of 2.5–10 mg following intravenous administration. It was demonstrated by the evidence that $C_{\text{max}}$ increased in a dose-proportional manner and $T_{1/2}$ presented consistent among the groups. Estimates of $AUC_0-\infty$ and $Cl$ were demonstrated to be dose-dependent but not exactly dose-proportional over the dose range, because of the lack of quantifiable concentrations in the terminal elimination phase at 2.5 mg group. Half-lives of therapeutic antibodies tend to be shorter for murine and chimeric (~2–3 d and ~8–10 d, respectively) compared with humanized (~20–23 d) products. The rapid elimination of murine IgGs from human circulation is in part attributed to lack of binding to human neonatal Fc receptor (FcRn) of the reticuloendothelial system (RES), which functions as a salvage receptor regulating IgG catabolism. With the protection of FcRn, humanized antibodies had longest half-lives due to the highest binding affinity for FcRn. Thus, it was not surprising that hu12F6mu, the modified version of humanized version of 12F6 antibody, decreased more quickly than the parent humanized antibody. The low ability of hu12F6mu binding to Fc receptor-bearing cells also likely contributed to the lack of an observed release of cytokines such as IFN-γ, TNFα and IL-6.

Immune suppression by anti-CD3 antibodies is thought to be mediated by depletion of circulating T cells. The infusion of hu12F6mu resulted in depletion of T cells from the peripheral circulation at a dose as low as 2.5 mg. T-cell depletion was observed within 6 h of hu12F6mu infusion and the duration and the extent of T-cell depletion was dose-dependent. At the lowest dose level (2.5 mg), recovery of T-cell levels occurred within 4–5 d, while the time to T-cell recovery was 8–10 d at the highest dose levels (10 mg). All T-cell subsets studied, including CD2+, CD3+, CD4+ and CD8+ cells were consistent in their depletion and recovery. In particular, the CD4+/CD8+ ratio remained within a normal range during and after recovery (data not shown).

| Lymphocyte subgroups | hu12F6mu dose | Baseline (Mean ± SD) | Minimal (Mean ± SD) | Min/Base (%) | $T_{1/2}$ (h) |
|----------------------|--------------|----------------------|---------------------|-------------|--------------|
| CD2+                 | 2.5 mg       | 68.19 ± 8.96         | 29.36 ± 11.53       | 43.1%       | 6 (2–24)     |
|                      | 5 mg         | 67.98 ± 4.70         | 19.62 ± 7.85        | 28.9%       | 6 (2–24)     |
|                      | 10 mg        | 64.96 ± 11.04        | 10.69 ± 8.17        | 16.5%       | 12 (2–48)    |
|                      | 2.5 mg       | 62.46 ± 11.43        | 12.86 ± 6.21        | 20.6%       | 6 (6–6)      |
| CD3+                 | 5 mg         | 59.92 ± 6.65         | 4.64 ± 2.67         | 7.7%        | 6 (2–24)     |
|                      | 10 mg        | 60.49 ± 10.58        | 1.96 ± 2.98         | 3.2%        | 6 (0.5–36)   |
| CD4+                 | 2.5 mg       | 34.49 ± 5.04         | 5.74 ± 2.98         | 16.6%       | 6 (2–24)     |
|                      | 5 mg         | 35.59 ± 6.39         | 3.28 ± 2.26         | 9.2%        | 6 (2–12)     |
|                      | 10 mg        | 36.32 ± 8.70         | 1.96 ± 1.26         | 5.4%        | 6 (2–12)     |
| CD8+                 | 2.5 mg       | 27.54 ± 11.32        | 12.56 ± 9.66        | 45.6%       | 6 (1–24)     |
|                      | 5 mg         | 25.79 ± 4.21         | 4.80 ± 2.37         | 18.6%       | 2 (1–12)     |
|                      | 10 mg        | 18.30 ± 5.18         | 3.78 ± 2.24         | 20.7%       | 6 (1–24)     |

Figure 7. The concentration-effect plots display the coherence between hu12F6mu serum concentration and CD3+ cell proportion (%) after administration of 10 mg of antibody. ■, CD3+ proportion (%); ●, hu12F6mu serum concentration (ng·L⁻¹).

CD3+ cell number recovered about half of the baseline level at the concentration of ~1,000 ng·mL⁻¹ (Fig. 7) in 10 mg dose group. There was no evidence to explain the relationship between CD3+ cell numbers and CD3 modulation. Cui proposed that the fluorescent intensity of the CD3 was an index of the function of CD3+ T cell. In other words, although the fluorescent of the CD3 was detected by flow cytometry, the function of these CD3+ cell might have been impaired as the fluorescent intensity decreased. For this reason, we deduced that 90% CD3 modulation could still be achieved at the concentration of ~1,000 ng·mL⁻¹ in 10 mg dose group. Further clinical trials should be carried out and clinical index such as acute rejection incidence rate should be adopted to evaluate the efficacy of hu12F6mu.

Previous evidence suggests that multiple mechanisms are involved in the immune suppression induced by anti-CD3 mAb depletion of peripheral blood, including TCR internalization, anergic T-cell induction, T-cell apoptosis, cytokine derivation and regulatory T-cell activation. OKT3 proved to possess the ability to deplete peripheral blood T cells within minutes to hours after an initial dose, which was also observed for...
hu12F6mu in our present study. In the early period of treatment with OKT3, a significant percentage of CD4+ and CD8+ T cells reappeared and only a low density of CD3 molecules were detected, as a result of CD3 modulation.\textsuperscript{31,32} Therefore, it could be concluded that both depletion and modulation contributed to the decline of T-cell subpopulations in the OKT3 treatment; however, from the data in this single dose study, it is difficult to draw a conclusion as to whether modulation, depletion or both resulted in decline of T-cell subpopulations. We will investigate the mechanisms of action of hu12F6mu, including T-cell redistribution, complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), apoptosis, in the ongoing multi-dose study and in vitro experiments.

The hu12F6mu mAb was specifically developed to reduce or eliminate humoral response. Anti-hu12F6mu antibodies were not detected in this study, which suggests that the humoral response to hu12F6mu may be substantially reduced compared with the response to murine 12F6. This property may provide an additional advantage with hu12F6mu re-treatment.

In summary, this study suggests that hu12F6m is well-tolerated and possesses potent pharmacological activity with respect to depletion of circulating CD3+ T cells. The results indicate that additional studies to define the mode of action, safety and clinical utility of hu12F6mu as a treatment and prophylactic in allograft rejection are warranted.

Materials and Methods

Drug. The hu12F6mu formulation was manufactured by National Engineering Research Center of Antibody Medicine (Shanghai). Hu12F6mu is a humanized anti-CD3 antibody with a mutated Fc (L234A, L235A). It is produced by recombinant DNA technology in the CHO cell expression system. The hu12F6mu formulation is supplied as a clear, sterile, colorless concentrate for further dilution and intravenous administration. Following reconstitution with 1 mL of sterile water, the solution is clear and colorless to pale yellow with a pH of 7.0. Each single use vial contains 5 mg hu12F6mu, 10 mg sucrose, 0.5 mg sodium phosphate dibasic heptahydrate and 0.2 mg polysorbate 80. No preservatives are added.

Study design and patients. This study was designed as a single-center, open-label dose escalation clinical trial to investigate the tolerability and PK of the test drug. The whole study was carried out in compliance with the ethics principles of the Declaration of Helsinki and Good Clinical Practice (GCP) guidelines. The study protocol was approved by the ethics committee of First Affiliated Hospital of college of Medicine, Zhejiang University. All subjects signed the informed consent forms before participation.

Adults, age range from 18–60 years, receiving primary renal allografts from cadaveric donors or human leukocyte antigen (HLA)-identical living donors, were eligible for the study. Patients were excluded if they had received multiple organ transplants or were previously exposed to CD3-specific mAbs. Patients with other significant diseases or infections were also excluded. Twenty-seven subjects were assigned randomly into three groups (nine subjects each). Every subject was randomly arranged to receive intravenous infusion of 2.5, 5 and 10 mg. The medication was diluted to a total volume of 100 mL with 0.9% sodium chloride solution and infused intravenously within 30 min. Physical examinations and laboratory tests including blood routine test, serum chemistry test and urine analysis were performed in pre-administration and 1, 3, 5, 7, 14, 21, 28, 35, 42, 49 d after medication administration.

Sample collection. Venous blood samples for analysis of hu12F6mu antibody concentration and lymphocyte subsets were drawn before drug administration; 0 and 30 min after discontinuation of the infusion began, and 2, 6, 12, 24 h after the infusion ended. Blood samples were also drawn 2, 3, 5, 7, 10, 14, 21, 28, 35, 49 d after the infusion ended. In total, 5 mL whole blood was drawn each time. For each sample, serum was harvested by means of centrifugation from 3 mL whole blood and stored for analysis at -20°C. The sample was also used to determine the induction of anti-hu12F6mu and neutralizing anti-antibody antibody. For each lymphocyte subset analysis, 2 mL whole blood sample was collected in anti-coagulated tubes and the samples had to be stained and detected by FCM within 24 h.

Hu12F6mu serum level assay. Serum levels of hu12F6mu were measured using a double-sandwich enzyme-linked immunosorbent assay (ELISA). The hu12F6mu standard and human CD3 ε0 fusion protein were provided by Shanghai Key Lab of Cell Engineering and Shanghai National Engineering Research Center for Antibody Medicine. A human CD3 ε0 was used as the solid-phase capture reagent in Nunc-Immuino Maxisorp 96-well microplates (Nalge Nunc International, Rochester, NY, USA). Binding of hu12F6mu to a coated well was detected using a detection antibody, a horseradish peroxidase (HRP)-conjugated sheep antihuman IgG reagent. In detail, plates were coated and incubated with human CD3 ε0 at a concentration of 1 mg·L\textsuperscript{-1} (100 μL/well) or buffer alone at 4°C overnight. The solution was aspirated and the micro titer plates were blocked with the addition of 350 μL/well of blocking solution (3% BSA in phosphate solution). After washing four times with Tris-buffered saline with 0.1% Tween 20, diluted standards (500–0.24 μg·mL\textsuperscript{-1}) were added at 100 μL/well, followed by the addition of 100 μL/well of detection antibody and incubation for 2 h at room temperature with continuous shaking. The microtiter plates were then washed three times again with Tris-buffered saline, and the bound HRP conjugate was detected with a peroxidase reagent. Calibration standards and QC samples were run four times, and samples were run twice. Hu126Fmu serum concentrations in each sample were calculated through a standard curve measured at an optical density of 450 nm (OD\textsubscript{450}) with reference at 630 nm using a plate reader. The following equation established from a 4 parameter logistic model was used as follows: \begin{equation} Y = (A_0 - A_1)/(1 + ([X/X_0]^n + A_0)\] + A_1, where X represents the concentration of hu12F6m in the samples, Y represents the absorbance at 450 nm, P represents the slope of the logit-log plot, X_0 represents the half-maximal effective concentration (EC_{50}) and A_0 and A_1 were the maximal and the minimal absorbance, respectively. The calculated value of...
Peripheral blood T-cell monitoring. Circulating peripheral blood T cells were monitored by flow cytometric analysis. mAbs used for immunophenotyping included the following (all purchased from Becton Dickinson): mouse anti-human CD2-PE (IgG1), mouse anti-human CD3-FITC (IgG1), mouse anti-human CD3-PC (IgG1), mouse anti-human CD4-FITC (IgG1) and mouse anti-human CD8-PE (IgG1). FITC-dimethylfumarimide was added to purified mAb at 1:10 weight/weight and incubated at 25°C for 4 h, followed by dialysis into phosphate-buffered saline containing an anion exchange resin [AG1-X8, 200–400 mesh, chloride form (Bio-Rad, New York, NY, USA)]. Aggregates were removed by centrifugation. Staining of peripheral blood lymphocytes (PBL) with fluorochrome-labeled mAbs was performed in whole blood and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Cytokine assay. IFNγ, TNFα and IL-6 levels in serum samples from hu12F6mu-treated patients were quantitated using commercial ELISA-based kits (R&D systems, Category No DIF50, Category No DIF50 DTA00C and Category No DIF50 D6050, respectively).

Anti-hu12F6mu antibody ELISA. The presence of serum anti-hu12F6mu antibody formation was monitored using a double-antigen ELISA. Hu12F6mu was used as the solid-phase capture reagent, and binding of anti-hu12F6mu antibodies to the coated and blocked wells was detected using an HRP-conjugated anti-hu12F6mu antibody. Colorimetric measurement and data analysis were performed as above. Regression of sample optical densities from a calibration curve using rabbit anti-hu12F6mu antibodies results in a quantitative range of approximately 160–20,000 ng·mL⁻¹ anti-hu12F6mu idiotype equivalents. When the samples were positive, neutralizing anti-antibody antibody would subsequently be detected by mixed lymphocyte reaction method.

Data analysis. The individual PK parameters were calculated by DAS 2.1 software (Wan nan Medical College, Wuhu, China) using non-compartment methods of analysis. Elimination half-life (τ1/2), elimination rate constant (K), mean retention time (MRT), clearance rate (CL) and apparent volumes of distribution (V) were obtained through this software. The area under the concentration-time curve (AUC) was calculated using the trapezoidal method. Maximal serum concentration (Cmax) and time to reach peak concentration (Tmax) of hu12F6mu were defined as observed. Serum drug level below the level of quantification (5 ng·mL⁻¹) was deemed equal to zero for this calculation.

Statistical analysis. Statistical analysis was performed with SPSS 13.0 software for Windows operation system (SPSS Inc., Chicago, IL, USA). All grouped data were expressed as means ± SD. For data among at least three groups, analysis of variance (ANOVA) was applied to test differences of data on normal distribution, and the Kruskal-Wallis test (K-W H test) was used when the data were not normally distributed. For a difference between two groups, the t-test was employed when the data were of normal distribution and variance was homogenous; otherwise, the Wilcoxon test was used. A p value <0.05 was considered statistically significant.

Acknowledgements
This work was supported by grants from National Natural Science Foundation of China, Ministry of Science & Technology of China (973 and 863 program projects), State Key Project for New Drug Development and Infectious Diseases and Shanghai Commission of Science & Technology (Key Laboratory and Projects).

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