Safety evaluation and pharmacokinetics of a novel human tumor necrosis factor-alpha exhibited a higher antitumor activity and a lower systemic toxicity

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We had prepared earlier a prokaryotic-expressed tumor necrosis factor-alpha (TNF-α) mutant that exhibited a higher antitumor activity and a lower systemic toxicity compared with that of wild-type TNF-α in both syngeneic murine tumor models and human tumor xenografts models. For its clinical use as an antitumor agent, we evaluated repeated-dose toxicity, anaphylaxis, genetic toxicity, pharmacokinetic, and metabolism in different animals according to the criteria of the biological investigational new drug application. It was found to be safe at a dose of 4×10^6 IU/kg/day for 60 days after administration in rhesus monkeys, but the TNF-α antibody level and liver toxicity needed to be monitored. No systemic anaphylaxis or genetic toxicity was found and the pharmacokinetic characteristics of the recombinant mutated human TNF-α (rmhTNF-α) were suited for clinical use. More than 96.3% of rmhTNF-α could be reclaimed from the urine and feces in 24 h after administration, which indicated the main excretion route. The results proved that the characteristics of this rmhTNF-α satisfied clinical trial requirements. The related positive clinical trial results will be reported in future. This study of novel rmhTNF-α is of considerable importance, not only given the proven usefulness of TNF-α local application therapies under isolated limp perfusion and isolated hepatic perfusion conditions for selected indications, but also implicated for systemic application of TNF-α. *Anti-Cancer Drugs* 21:243–251 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Tumor necrosis factor-alpha (TNF-α, cachectin) is a pleiotropic cytokine with a variety of biological activities, including cytotoxicity, immune cell proliferation, and mediation of inflammatory responses [1]. By means of receptor-dependent apoptosis or receptor-independent cytotoxic activity, TNF-α can inhibit a wide range of human and murine tumor cell lines and also tumor microvasculature endothelial cells *in vivo* and *in vitro* [2–6]. In 1975, Dr Old's research group isolated this cytokine as a serum factor; the remarkable ability of TNF-α, especially in combination with interferon, mediates hemorrhagic necrosis of certain tumors to an extent that is so far unmatched by any other cytokines [7–11]. Unfortunately, as TNF-α has also been implicated in various functions in immune and inflammatory responses, its clinical use for systemic treatment is limited by proinflammatory side effects, including fever, dose-limiting hypotension, hepatotoxicity, intravascular thrombosis, and hemorrhage. Clinical trials in cancer patients estimated that a TNF-α dose would be effective at only 5–25 times the maximum tolerated dose [12–15].

Various strategies, such as small molecule inhibitor of apoptosis (IAP) antagonists synergism, prodrugs, and local application (isolated limb or hepatic perfusion), have been pursued to minimize the systemic toxicities of TNF-α, to increase TNF-α sensitivity and availability in tumor cells, and therefore to increase the therapeutic index [16–20]. A promising approach to achieve these aims is to design clinically applicable TNF-α mutants with low systemic toxicity and high efficiency and it has been of great interest [21–25]. On the basis of these works, we prepared a TNF-α mutant (recombinant mutated human TNF-α; rmhTNF-α) by deleting the first seven amino acids at the N-terminal, replacing Pro 8, Ser 9, and Asp 10 with Arg 8, Lys 9, and Arg 10, and at the C-terminal, Leu 157 with Phe 157. The potential clinical application is promising because of the much higher

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antitumor effects, and at least 50 times higher LD_{50} (50% lethal dose) of this prokaryotic expressed rmhTNF-α than native TNF-α on several different syngeneic murine tumors and human tumor xenografts in nude mice [26].

For further study and potential clinical application, repeated-dose toxicity, anaphylaxis, genetic toxicity, pharmacokinetic and metabolism of this mutant rmhTNF-α are investigated here in rodents and primates. The results of study and interrelated studies satisfied the requirements of later clinical trials. Studies on novel rmhTNF-α with low systemic toxicity and high efficiency are of considerable importance, not only given the proven usefulness of TNF-α local application therapies under isolated limp perfusion (ILP) and isolated hepatic perfusion (IHP) conditions for selected indications, but also implicated for the systemic application of TNF-α.

Materials and methods

Test article

The TNF-α mutant (recombinant mutated human TNF-α; rmhTNF-α) was prepared by protein engineering [26]. The rmhTNF-α was purified to 97.5% by high-performance liquid chromatography with a $0.1–1.2 \times 10^9$ IU/mg bioactivity estimated by standard procedures on the mouse fibroblast cell line, L929, formulated with 3% mannitol, sterilized, and lyophilized meeting regulatory requirements of preclinical studies.

Animals

ICR mice were 8–12 weeks old and weighed between 18 and 22 g. Sprague–Dawley rats were approximately 6 weeks old and weighed about 220 g. New Zealand white rabbits were 13–17 weeks old and weighed between 2.4 and 3.6 kg. Guinea pigs were 8–10 weeks old and with an average body weight of 260 g. Rhesus monkeys, *Macaca Speciosa Thibetanas Milne-Edwards*, were 3–5 years old and weighed between 4 and 6 kg. All of the rodents were purchased from Experimental Animal Center of Lanzhou Medical University (Lanzhou, China). The rhesus monkeys were purchased from Medical Science Experimental Animal institute of Sichuan (Chengdu, China).

The animals were maintained in an air-conditioned barrier-system animal room at an ambient temperature of 25 ± 2°C, a relative humidity of 50 ± 10%, and a 12 h on/off light cycle. All the animals were treated humanely, and the study protocols were in accordance with the Regulations of Good Laboratory Practice for nonclinical laboratory studies of drugs issued by the National Scientific and Technologic Committee of People's Repubilc of China.

Repeated-dose toxicity

Ten female and 10 male rhesus monkeys were grouped to receive three doses of rmhTNF-α [2×10^5, 2×10^6, and 4×10^6 IU/kg/day for 60 days, intramuscular (i.m.), which were 5, 50, and 100 times higher than the clinical dose for adults]. TNF-α at 4×10^6 IU/kg/day and saline were used as controls. The general condition and behavior of all animals were checked daily. The following parameters were evaluated at days 7, 14, 30, and 60: general signs, food consumption, body weight, electrocardiogram, ophthalmology, hematology, hemostasis, clinical chemistry, urinalysis, and anti-TNF antibody level. After 60 days, half of the experimental animals were killed for necropsy. The remaining were raised for another 28 days for further observation and then underwent autopsy.

Local tolerance studies and sensitization response

Local muscular irritation of rmhTNF-α was evaluated after a single i.m. dose in four New Zealand white rabbits (two males and two females). The dose of 4×10^6 IU/kg rmhTNF-α (1 ml) was injected into the right thigh muscle of the rabbits, and saline as the negative control was injected into the corresponding muscle of the left leg. The animals were killed for necropsy at 48 h after administration, and the muscles of injection sites were examined macroscopically and histopathologically.

Twelve guinea pigs received 2.5×10^5 IU/pig rmhTNF-α i.m. every 2 days for three times. As a challenge, 5×10^5 IU rmhTNF-α was administered intravenously (i.v.) 14 days later ($n=6$) or 21 days later ($n=6$). Saline and bovine serum albumin (BSA, 5 mg/pig/time, 10 mg/pig challenge) were used as blank and positive controls. Hypersensitivity responses, such as cough, dyspnea, spasm and even death, were monitored daily and recorded.

Genetic toxicity

Bacterial reverse mutation tests (Ames assay)

The mutagenic activity of rmhTNF-α was assessed in four *Salmonella typhimurium* mutant strains, TA_{97}, TA_{98}, TA_{100}, and TA_{102}. A serial dilution of rmhTNF-α was prepared and tested at five concentrations ranging 5×10^5, 2.5×10^6, 5×10^6, 2.5×10^7, 5×10^7, 5×10^8 IU for each plate. Negative (sterile water) and positive controls (2-aminofluorene, daunorubicin, MMS and 1,8-dihydroxyanthraquinone) were run simultaneously with the test. Reverse mutation clones were counted and every sample was made in triplicate.

Bone marrow micronucleus assay

The activity of rmhTNF-α to induce bone marrow micronucleus was assessed in the ICR strain mice. The first three groups (six mice/group) received rmhTNF-α by i.m. injection at doses from 3.5×10^8, 0.7×10^8 to 0.14×10^8 IU/kg. Saline and cyclophosphamide (30 mg/kg) were used as negative and positive controls. Forty-eight hours later, the animals were killed and the thighbone marrow cells were analyzed following methanol fixing and Giemsa staining. The frequency of micronuclei was counted based on an examination of 1000 polychromatic erythrocytes for every mouse.

Chromosome aberration assay

The potential of rmhTNF-α to induce chromosome aberrations was tested in cultured Chinese hamster lung (CHL) cells. rmhTNF-α ranging 3125, 6250, 12 500, 25 000, 50 000 IU/ml were applied to CHL cells for 46 h. After colcemid arresting, methanol fixing, and Giemsa staining, the percentage of CHL cells with chromosome aberrations was determined by examining 100 cells in every slide.

Teratogenicity test

Three doses of rmhTNF-α (2.5×10^8, 0.5×10^8, and 0.1×10^8 IU/kg, i.m.) were injected into three cohorts of pregnant rats (20 animal/cohort) on the 6th and 15th days after of pregnancy. Normal saline and *N,-N'*-methylenebis (1 mg/kg) were used as negative and positive controls, respectively. On the 19th day of pregnancy, exterior examination and skeleton examination of all fetuses were carried out after euthanasia.

Pharmacokinetic studies in mice with radioactive-labeled rmhTNF-α

rmhTNF-α was labeled with iodine-125 and purified by S-200 exclusion chromatography (AKTA explorer; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). The [^{125}I]-rmhTNF-α reaction product was determined to have high radiochemical purity (> 95%) and specific radioactivity of 43 μCi/μg with 1×10^9 IU/mg bioactivity, which was estimated by standard procedures on L929.

ICR mice were grouped to receive three doses of [^{125}I]-rmhTNF-α (10, 20, and 40 μg/kg, i.m.). Every mouse received a single intraperitoneal (i.p.) dose of 0.2 ml NaI (100 mg/ml) before administration. Another group received i.v. dose of [^{125}I]-rmhTNF-α at a concentration of 10 μg/kg. Blood samples were collected from replicate animals (n=4, two males and two females) in each group at time points of 0.25, 0.5, 1, 2, 3, 4, 6, 8, 16 h after injection through the fossa orbitalis and the serum was divided. Each serum sample (10 μl) was tested with a SECS3000 chromatography column (PHenomenex, Torrance, California, USA) and eluted with 0.05 mol/l phosphate buffer, pH 6.9, at a flow rate of 1 ml/min in SP100 high-performance liquid chromatography system (SIELC Technologies, Prospect Heights, Illinois, USA). The radioactivity of the collected fractions within the settled time was quantified in duplicate by LS 6500 liquid scintillation spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). The ^{125}I content of each sample was adjusted for sample volume and the concentration of prototype rmhTNF-α in each sample was determined according to the radioactivity–concentration standard curve.

Pharmacokinetic studies in rhesus monkeys with enzyme-linked immunosorbent assay

Four rhesus (two females and two males) monkeys received a single i.m. administration of rmhTNF-α (dissolved in PBS) at a dose of 40 μg/kg. Blood samples were collected at time points of 1, 2, 3, 4, 6, 8, 10 h postdosing through the saphenous vein of the hind limb and the serum was divided.

Two TNF-α mousemonoclonal antibodies against different epitopes (kindly provided by the Immunology Department, the Fourth Military Medical University, Xi'an, China) were used as capture and detecting antibody. Horseradish peroxidase-labeled anti-mouse IgG (Biotec, Dalian, China) and ABTS system (Sigma, St. Louis, Missouri, USA) were used to develop a reaction with a Bio-Rad ELISA reader (450 nm). The concentrations of rmhTNF-α in serum samples were determined according to the absorbance and concerted standard curve.

Tissue distribution and metabolism studies

For tissue distribution and metabolism studies, ICR mice were housed in metabolic cage units with free access to food and water. Urine and feces were collected. Each mouse received [^{125}I]-rmhTNF-α administration (10 μg/kg, i.m.) after a single i.p. dosing of 0.2 ml NaI (100 mg/ml, i.p.). At 0.5, 1, 2, 3, and 8 h postdosing, animals (n=6 per time point) were killed, and blood samples and selected tissues were collected. One hundred milligrams of every selected tissue (brain, heart, kidneys, liver, spleen, lung, stomach, intestine, muscle, grease, ovaries, womb, testicles) was homogenized to yield serum (the actual weight was recorded if it did not reach 100 mg). The radioactivity of the 20 μl of serum was quantified in duplicate by liquid scintillation spectrophotometer. The radioactivity of the urine and the feces collected over the following periods: 0–2, 2–4, 4–8, 8–12, and 12–24 h postdosing (n=4, two males and two females) were quantified by the same method. The corresponding quantity of prototype rmhTNF-α was decided by the radioactivity and radioactivity–concentration standard curve.

Statistical analysis

The Student's t-test was used to determine the significance between each experimental group according to the DAS software developed by the Chinese Society of Pharmacology (Beijing, China). The difference was considered statistically significant when P value was less than 0.05.

Results

Repeated-dose toxicity study in monkeys

Similar to the former results [26], all monkeys in the TNF-α treatment group had severe edema in the perineum, abdomen, and extremitas inferiors; however, only two monkeys in the rmhTNF-α cohorts had minimal edema in the perineum and the eyelids. The edema of all animals disappeared after 28 days of recovery. From the results of the hematological tests and clinical chemical analyses, the decline of erythrocyte, hemoglobin,

leukocyte blood disk, albumin, and creatinine plasma levels was found in the TNF-α treatment group ($P < 0.05$) but not in three rmhTNF-α groups. Neither rmhTNF-α at various doses nor TNF-α had negative effects on the monkeys' blood pressure and heart rate. No abnormalities were observed in routine urinalysis of every cohort.

Either rmhTNF-α or TNF-α treatment animals showed a high level of TNF-α antibody during the 15th through the 30th day of administration (Fig. 1). After the end of the treatment, antibody response recovered without additional care. Inflammatory cell infiltration and mild atrophy at the center of the liver, mild colitis, enteritis, inflammation in pulmonary interstitial, mild gliocyte hyperplasia, and striated muscle necrosis of the injection site were found in the autopsy of TNF-α treatment animals. Only mild inflammatory cell infiltration at the center of the liver was found in rmhTNF-α cohorts.

Local tolerance studies and sensitization response

There were no hyperemia, hydropsia, necrosis, and treatment-related clinical or histopathological findings at the injection sites in the rabbits. There were no symptoms of allergic response in the guinea pigs receiving systemic administration of rmhTNF-α. The positive control, BSA, caused cough, spasm, tic, shock, and even death in guinea pigs.

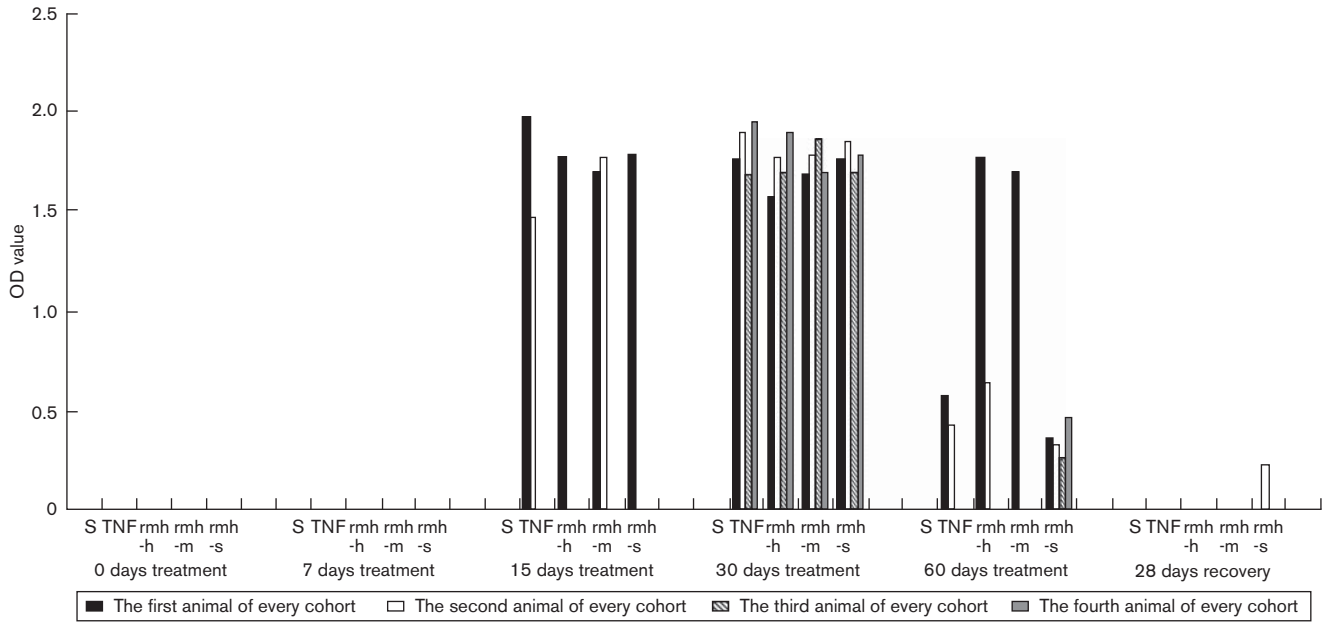
Genetic toxicity

The results of rmhTNF-α did not show any significant difference from that of saline in the Ames test (Table 1), the bone marrow micronulecus assay (Fig. 2), or the chromosome aberration assay (Fig. 3), indicating that no genetic toxicity was found *in vitro*. In the teratogenicity tests, no fetal malformations such as umbilical hernia, tail absence, limb inversion, and limb eversion, which appeared in the methylenebis-treated cohort were found. There were also no abnormalities observed in the skeletal development of these fetuses compared with the 39.4% malformation rate for methylenebis treatment ($P < 0.01$).

RmhTNF-α serum pharmacokinetics in mice

Concentration–time data of i.m. administrations, as derived from radioactivity measurements, were fitted to a one-compartment pharmacokinetic model. Data of i.v. administration were fitted to a two-compartment pharmacokinetic model. According to the concentration change with time (Fig. 4), the rmhTNF-α could rapidly enter into circulation and the maximum serum concentration of rmhTNF-α could be reached in 0.5 h. Pharmacokinetic parameter estimates, as shown in Table 2, were derived using WinNonLin 5.0.1 (Pharsight Corporation, Cary, North Carolina, USA). Systemic clearance (CL) and area under serum concentration–time curve of i.m. administration show marked differences relative to

Fig. 1



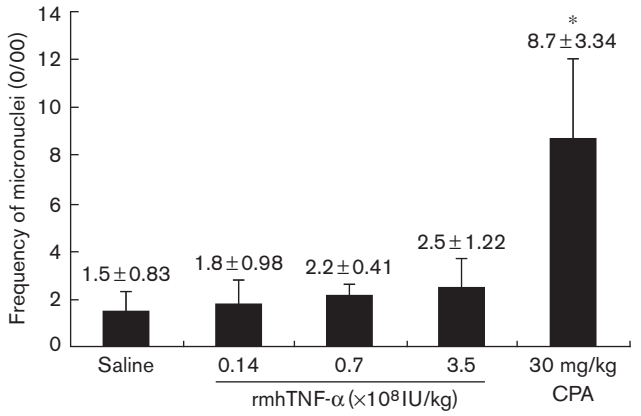
Changes in tumor necrosis factor (TNF)-α antibody level in monkey serum with recombinant mutated human TNF-α (rmhTNF-α) or TNF-α treatments in a repeated-dose toxicity study. Five cohorts of rhesus monkeys (two females and two males for every cohort) were treated with saline (S), TNF-α at 4×10^6 IU/kg/day (TNF), rmhTNF-α at 4×10^6 IU/kg/day (rmh-h), rmhTNF-α at 2×10^6 IU/kg/day (rmh-m) or rmhTNF-α at 2×10^5 IU/kg/day (rmh-s) for 60 days, respectively. The serum TNF-α antibody elicited by treatments were tested using a sandwich ELISA assay before initial dose, after 7, 15, 30, 60 days treatments, and 28 days recovery period. Either TNF-α or rmhTNF-α (at every dose) caused an immunoreaction after 30 days treatment. The peak levels of the rmhTNF-α and TNF-α antibody in animal serum appeared during the 15th through the 30th day of administration. The antibody level decreased with the process of treatments. After 28 days recovery, only very low antibody levels could be detected in one monkey in the rmh-s cohort. OD, optical density.

Table 1 Reverse mutation clone quantities of *Salmonella typhimurium* strains treated with rmhTNF-α or mutagens (Ames assay)

	Reagent							
	rmhTNF-α ($\times 10^6$ IU/plate)							
Strains	Saline	0.5	2.5	5	25	50	500	Mutagen
TA97								
−S9	130±12	120±3	110±30	101±17	107±21	127±28	129±13	3079 (NF)*
+S9	149±4	139±9	138±5	131±13	130±7	139±9	157±15	3972 (2-AF)*
TA98								
−S9	32±2	29±4	33±0	32±2	30±3	30±2	32±5	1438 (DNR)*
+S9	35±2	35±7	34±2	33±4	37±2	38±4	32±2	3062 (2-AF)*
TA100								
−S9	164±7	158±11	160±16	139±5	157±14	185±42	185±42	3357 (MMS)*
+S9	189±6	170±3	183±5	167±6	175±17	177±13	164±3	2377 (2-AF)*
TA102								
−S9	218±24	258±12	273±5	280±11	264±11	266±9	290±0	3141 (NF)*
+S9	295±11	291±7	279±2	283±12	290±8	281±15	261±0	1738 (HDT)*

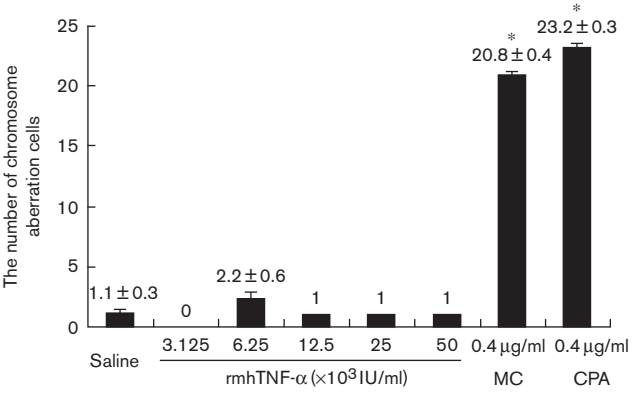
rmhTNF, recombinant mutated human tumor necrosis factor-α; S9: metabolic activation, a cofactor supplemented liver postmitochondrial fraction from Aroclor 1254-induced rats. NF (2,4,7,-trinitrofluorenone) 0.2 μg/plate; 2-AF (aflatoxin) 50 μg/plate; DNR (daunorubicin) 10 μg/plate; MMS (methyl methanesulfonate) 3 μg/plate; HDT (hydroxyanthraquinone) 80 μg/plate.
*$P<0.01$.

Fig. 2



Frequency of micronuclei in mouse polychromatic erythrocytes after recombinant mutated human tumor necrosis factor-α (rmhTNF-α) treatments (0/00). Activity of rmhTNF-α to induce bone marrow micronucleus was assessed in ICR mice. Three groups (6 mice/group) received 3.5×10^8, 0.7×10^8 to 0.14×10^8 IU/kg intramuscular administrations of rmhTNF-α, respectively. Saline and cyclophosphamide (CPA, 30 mg/kg) were used as negative and positive controls. Forty-eight hours later, the animals were killed and the thighbone marrow cells were analyzed after methanol fixing and Giemsa staining. The frequency of micronuclei was counted based on an examination of 1000 polychromatic erythrocytes for every mouse. The results did not show any significant difference between rmhTNF-α and saline cohorts ($P>0.05$), which indicated no micronuclei inducing toxicity for rmhTNF-α in mice. *$P<0.01$.

Fig. 3



Percentage of Chinese hamster lung (CHL) cells with chromosome aberrations induced by recombinant mutated human tumor necrosis factor-α (rmhTNF-α) (*$P<0.01$). The highest doses of rmhTNF-α, the doses of mitomycin C (MC) and cyclophosphamide (CPA) used in this experiment were 50% LD50 (the median lethal dose) of every reagent against CHL cells. There were no significant differences between rmhTNF-α groups and saline. MC and CPA used as positive controls had over 20 times more toxicity than rmhTNF-α and saline.

i.v. Compared with i.v. administration, the absolute bioavailability of i.m. administration of rmhTNF was 0.211.

RmhTNF-α serum pharmacokinetics in rhesus monkeys

The time for rmhTNF-α to reach its maximum serum concentration after administration differed greatly among monkeys. The average time was 1.7 h. The maximum serum concentrations of the animals ranged from 575 to 1120 pg/ml, which also varied greatly (Fig. 5). The elimination of rmhTNF-α in rhesus monkeys was also evaluated with the one-compartment model. The average half-life in circulation was 1.9 h (Table 3).

Tissue distribution and metabolism studies

According to the tissue distribution results (Fig. 6), rmhTNF-α could enter different tissues rapidly in half an hour after i.m. administration. Except in the serum, the drug concentration in the kidney was the highest compared with other tissues. The drug concentration was lowest in the brain. The stomach and intestine also showed rmhTNF-α distribution. The rmhTNF-α was mainly excreted renally and 86.8% of the prototype

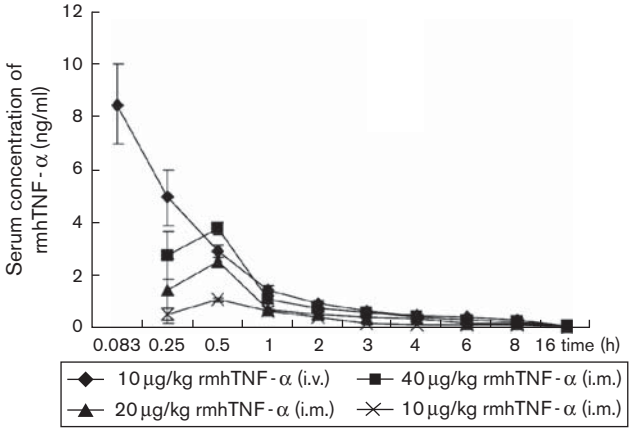
reagent could be reclaimed in urine 24 h after administration (Table 4). With 9.5% rmhTNF-α reclaimed in feces, the total amount could reach 96.3% in 24 h, which indicates the metabolism route of the rmhTNF-α.

Discussion

Designing a clinically relevant TNF-α mutant with low systemic toxicity and high antitumor activity has been of intense pharmacological interest in the past two decades [20–25]. Human TNF-α, which binds to the murine TNF receptor 55 (TNF-R55, 55 kDa), but not to the murine TNF receptor 75 (TNF-R75, 75 kDa), exhibits retained antitumor activity and reduced systemic toxicity in mice compared with that of murine TNF-α, which binds to both murine TNF receptors [27,28]. On the basis of these results, many TNF-α mutants that selectively bind to TNF-R55 have been designed [22,29]. Nevertheless, TNF-R55, apart from its death domain, which indicates the apoptosis, can also induce NF-κB activation, especially in the presence of high concentrations of the TNF receptor associated factor 2, as such activating the expression of proinflammatory genes which indicates its great side effect profile for treatment [30,31]. At the same time, NF-κB activation can impair the TNF-α-induced apoptosis in cancer cells [32]. A TNF-α mutant that preferentially binds to TNF-R55 developed serious systemic toxicity in baboons and several observations that TNF-R75 induces apoptosis of the expressing cells independent of TNF-R55 suggests that the role of TNF receptors in the TNF-α treatment is multiple and needs to be investigated further [5,29,33,34].

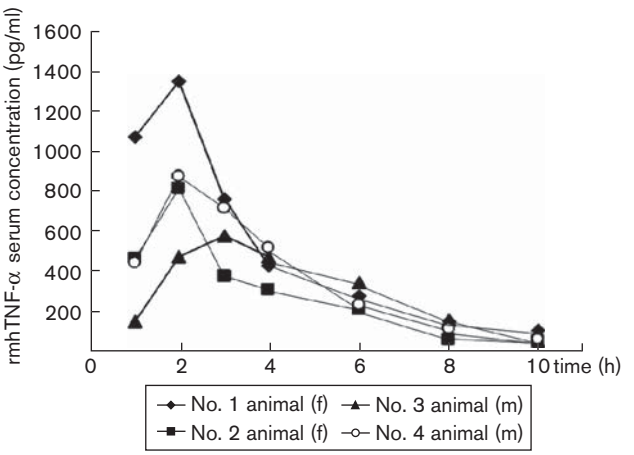
As the functions of TNF receptors and even the receptor-independent cytotoxic activity of TNF-α by ion channels formation [35] is complicated, the design of our rmhTNF-α is not based on receptor-selective binding ability, but on the fact that the increased basicity on the *N*-terminal can

Fig. 4



Serum concentration time course of three intramuscular (i.m.) doses (10, 20, and 40 μg/kg) or one intravenous (i.v.) dose (10 μg/kg) of [^{125}I]-recombinant mutated human tumor necrosis factor-α (rmhTNF-α) administration in mice. Serum from replicate animals ($n=4$) in each group were collected at time points of 0.25, 0.5, 1, 2, 3, 4, 6, 8, 16 h postdosing. The radioactivity of serum samples (after high-performance liquid chromatography purification) was quantified in duplicate by liquid scintillation spectrophotometer. The concentration of prototype rmhTNF-α in each samples were determined according to the radioactivity–concentration standard curve. Pharmacokinetic profiles characterized by rapid tissue distribution and the maximum serum concentration of rmhTNF-α could be reached in 0.5 h in case of i.m. doses.

Fig. 5



Serum concentration time course of recombinant mutated human tumor necrosis factor-α (rmhTNF-α) [40 μg/kg, intramuscular (i.m.)] in rhesus monkey. Four rhesus [two females (f) and two males (m)] monkeys received a single i.m. administration of rmhTNF-α (dissolved in PBS) at a dose of 40 μg/kg. Blood samples were collected at time points of 1, 2, 3, 4, 6, 8, 10 h postdosing through the hind limb saphenous vein and the serum was divided. The concentrations of rmhTNF-α in serum samples were determined by enzyme-linked immunosorbent assay. The time for rmhTNF-α to reach its maximum serum concentration after administration differed greatly among monkeys. The average time was 1.7 h. The maximum serum concentrations of the animals ranged from 575 to 1120 pg/ml, which also varied greatly. The elimination of rmhTNF-α in rhesus monkeys was also evaluated with the one-compartment model. The average half-life in circulation was 1.9 h.

Table 2 Serum pharmacokinetic parameters of [^{125}I]-rmhTNF-α after three i.m. doses (10, 20, and 40 μg/kg) or one i.v. dose (10 μg/kg) injection in mice

	α (/h)	β (/h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	K_α (/h)	$t_{1/2\ K\alpha}$ (h)	T_{peak} (h)	V_d (l/kg)	C_{max} (ng/ml)	CL (l/h/kg)	AUC (ng h/ml)
10 μg/kg (i.v.)	3.79	0.26	0.18	2.10	–	–	–	0.91	–	1.18	8.46
10 μg/kg (i.m.)	–	0.72	–	0.98	3.31	0.21	0.59	7.72	0.84	5.56	1.79
20 μg/kg (i.m.)	–	1.01	–	0.69	4.89	0.14	0.41	7.04	1.88	7.07	2.83
40 μg/kg (i.m.)	–	1.16	–	0.60	6.47	0.11	0.32	8.73	3.14	10.14	3.94

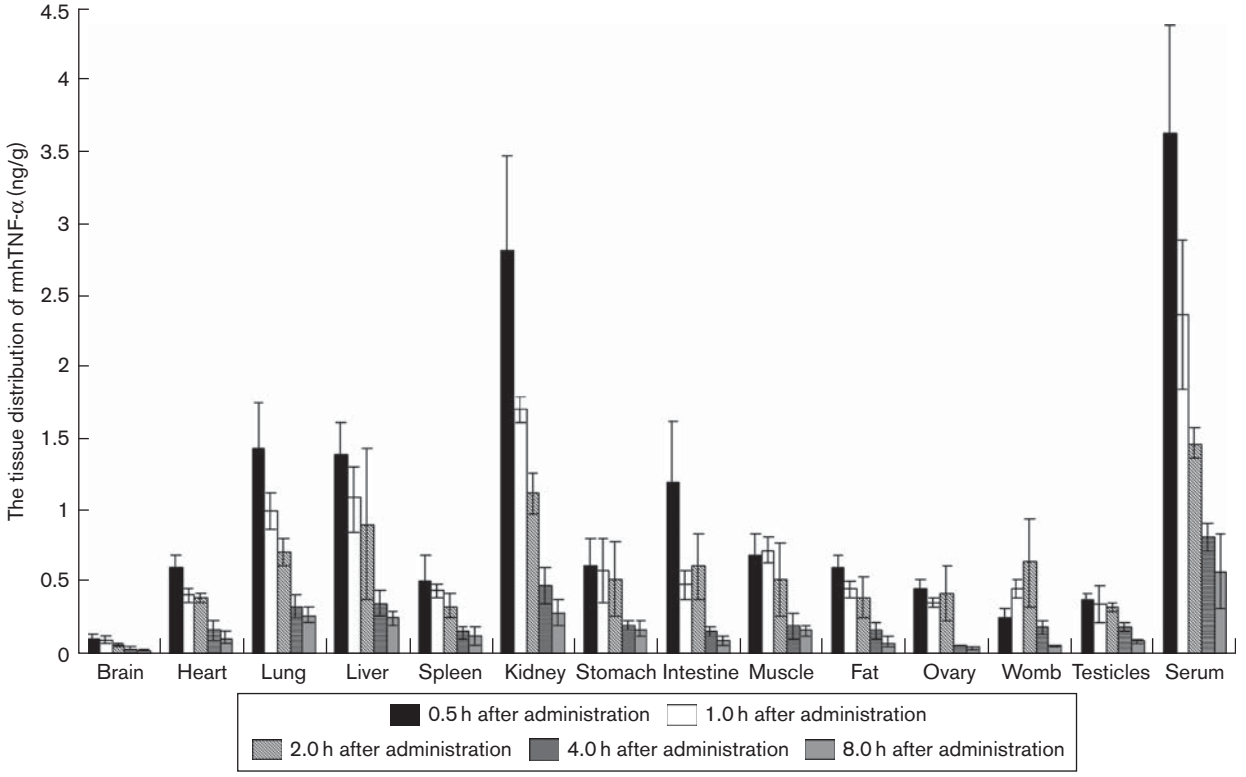
AUC, area under serum concentration–time curve; CL, systemic clearance; i.m., intramuscular; i.v., intravenous; rmhTNF, recombinant mutated human tumor necrosis factor-α.

Table 3 Serum pharmacokinetics parameters of rmhTNF-α (40 μg/kg) in rhesus monkey tested with sandwich enzyme-linked immunosorbent assay

	$t_{1/2\ K\alpha}$ (h)	T_{peak} (h)	V_d (l/kg)	C_{max} (pg/ml)	CL (l h/kg)	AUC (pg h/ml)
The first animal (f)	2.35	0.99	27	1119	8	5082
The second animal (f)	2.02	1.34	46	744	16	2536
The third animal (m)	1.42	2.69	28	575	13	2967
The fourth animal (m)	1.90	1.84	30	895	11	3612
X ± s	1.92 ± 0.39	1.72 ± 0.74	32.75 ± 8.92	833 ± 231	12 ± 2.92	3549 ± 1113

AUC, area under serum concentration–time curve; CL, systemic clearance; f, female; m, male; rmhTNF, recombinant mutated human tumor necrosis factor-α.

Fig. 6



Tissue distribution profiles after one intramuscular dose of [^{125}I]-recombinant mutated human tumor necrosis factor-α (rmhTNF-α) (10 μg/kg) in mice. Blood and selected tissues (100 mg for every kind) were collected at 0.5, 1, 2, 3 and 8 h postdosing (n = 6 per time point). The radioactivity of the 20 μl serum of every selected tissue (yielded by homogenizing) was quantified in duplicate by liquid scintillation spectrophotometry. The corresponding quantity of prototype rmhTNF-α in tissue was determined by radioactivity and the radioactivity–concentration standard curve. Except in the serum, the drug concentration in the kidney was the highest compared with other tissues. The drug concentration was lowest in the brain. There were also comparatively high distributions in the lung, the liver, and the intestine.

Table 4 Metabolism profile of one intramuscular dose of [^{125}I]-rmhTNF-α (10 μg/kg) in mice

	Urine			Feces		
Sample period (postdose) (h)	Amount (ng/mouse)	Accumulated amount (ng/mouse)	Excretion rate (%)	Amount (ng/mouse)	Accumulated amount (ng/mouse)	Excretion rate (%)
0–2	107.1 ± 6.9	107.1 ± 6.9	53.55	7.1 ± 3.0	7.1 ± 3.0	3.55
2–4	35.1 ± 2.1	142.2 ± 4.5	71.10	6.9 ± 1.4	14.0 ± 2.2	7.00
4–8	22.8 ± 6.7	165.0 ± 5.2	82.50	2.9 ± 2.2	16.9 ± 2.2	8.45
8–12	3.2 ± 1.2	168.2 ± 4.2	84.10	0.7 ± 0.1	17.6 ± 1.7	8.80
12–24	5.4 ± 2.6	173.6 ± 3.9	86.80	1.4 ± 0.3	19.0 ± 1.4	9.50

rmhTNF, recombinant mutated human tumor necrosis factor-α.

significantly increase the cytotoxicity of the TNF-α on tumor cells and earlier studies on TNF-α mutants [25,36,37]. The N-terminus and C-terminus were all reconstructed by protein engineering, the resulting rmhTNF-α had a clearly higher antitumor effect than TNF-α on the mouse S180 sarcoma, H22 liver carcinoma, B16 melanoma xenografts in mice [26]. This rmhTNF-α has no preferential TNF-R55 binding selection. Between the rmhTNF-α-resistant and sensitive tumor cell lines, there was no difference between the TNF-R55 expression levels but a significant difference was observed in the activation of NF-κB [38]. We hypothesized that the mechanism of action of rmhTNF-α had no relationship with the binding ability of TNF-R55, but it may be correlated with the signaling pathway of NF-κB. This hypothesis is currently being studied.

For translating this rmhTNF-α into the clinic, we conducted preclinical safety studies in this study. In repeated-dose toxicity study, edema and liver toxicity induced by rmhTNF-α in monkeys were much lower than by wild-type TNF-α, which was consistent with former results. The lower edema induction of rmhTNF-α may be correlated with the mutation, enhancing the function of the lectin-like domain of TNF-α. This could activate sodium transport in alveolar epithelial cells, peritoneal macrophages, and microvascular endothelial cells, and thus activate edema reabsorption *in situ* [39]. Although the clinical chemical analyses showed the liver toxicity only in the TNF-α treatment cohort but not in the rmhTNF-α cohorts, a little inflammatory cell infiltration was found at the center of the liver after necropsy. Liver toxicity should still be noted during clinical use.

Induced antibody development is an inevitable problem in the development of biological products and there are no effective methods for control. Every dose of rmhTNF-α or TNF-α could induce high levels of TNF-α antibodies in monkeys after 30 days of treatments. The development of antibodies will decrease the efficacy of the reagents and may elicit anaphylaxis in hosts. Fortunately, the antibody levels decreased with treatment. It could not be detected in some animals after 60 days of administration. These results support continued monitoring of TNF antibody levels during the treatment and cessation should be considered when the antibody levels are too high.

The pharmacokinetics of rmhTNF-α was studied in the mice or monkeys. The maximum serum concentration of rmhTNF-α could be reached in 0.5 h after i.m. administration and the half-life was about 1 h in mice which was not lower than i.v. administration. These two times were all prolonged to 1.7 and 1.9 h in monkey, which indicated the longer functional time of rmhTNF-α in primate animals. The biodistribution of rmhTNF-α accorded with normal biological products, and the clearance route was mainly renal. In both mice and monkeys, more than 95% of rmhTNF-α could be reclaimed from the urine and the feces 24 h after administration, which indicated the main excretion route.

After the preclinical study of rmhTNF-α-satisfied clinical trial requirements, we conducted phase 1 and phase 2 clinical tolerance, and efficiency studies in five hospitals in China approved by the Ministry of Health and State Food and Drug Administration of the People's Republic of China. At single doses ranging from 2.5×10^5 to 4×10^6 IU/m^2/day, rmhTNF-α could be well tolerated with mild side effects, such as fever, fatigue, muscle soreness, and anorexia. Compared with the maximum TNF-α dose (1×10^6 IU/m^2/day) recommended by earlier references, the tolerance dose of rmhTNF-α was four times higher than TNF-α in tumor patients. The overall response rate of rmhTNF-α and chemotherapy combination in lung cancer patients is 48.71% (complete response + partial response, $P < 0.01$). We will report these results in future.

In summary, this prokaryotic-expressed recombinant-mutated human TNF-α (rmhTNF-α) has lower toxicity and higher efficacy in antitumor treatments than TNF-α. The preclinical animal studies promised its clinical trial worth and safety. In addition, TNF-α exerts antitumor effects by inducing apoptosis in not only tumor cells but also in endothelial cells in the microvasculature of tumors. To increase the therapeutic index of the rmhTNF-α, we coupled integrin $\alpha_v\beta_3$-targeted RGD4C peptide to the N-terminal of rmhTNF-α. This RGD4C-rmhTNF-α fusion protein has tumor-specific delivery ability [40]. This study provides more insight into the development of the RGD4C-rmhTNF-α or other TNF-α mutants as anticancer reagents.

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References

1 Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001; **104**:487–501.
2 Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. *Cell* 1993; **74**:845–853.
3 Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* 1999; **17**:331–367.
4 Chang MP, Wisnieski BJ. Comparison of the intoxication pathways of tumor necrosis factor and diphtheria toxin. *Infect Immun* 1990; **58**:2644–2650.
5 Lucas R, Garcia I, Donati YR, Hribar M, Mandriota SJ, Giroud C, *et al.* Both TNF receptors are required for direct TNF-mediated cytotoxicity in microvascular endothelial cells. *Eur J Immunol* 1998; **28**:3577–3586.
6 Slowik MR, Min W, Ardito T, Karsan A, Kashgarian M, Pober JS. Evidence that tumor necrosis factor triggers apoptosis in human endothelial cells by

interleukin-1 -converting enzyme-like protease-dependent and -independent pathways. *Lab Invest* 1997; **77**:257–267.
7 Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* 1975; **72**:3666–3670.
8 Asher A, Mule JJ, Reichert CM, Shiloni E, Rosenberg SA. Studies on the anti-tumor efficacy of systemically administered recombinant tumor necrosis factor against several murine tumors in vivo. *J Immunol* 1987; **138**:963–974.
9 Creasey AA, Reynolds MT, Laird W. Cures and partial regression of murine and human tumors by recombinant human tumor necrosis factor. *Cancer Res* 1986; **46**:5687–5690.
10 Sugarman BJ, Aqqarwal BB, Hass PE, Figari IS, Palladino MA Jr, Shepard HM. Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. *Science* 1985; **230**:943–945.
11 Fiers W. Tumor necrosis factor, Characterization at the molecular, cellular and in vivo level. *FEBS Lett* 1991; **285**:199–212.
12 Abbruzzese JL, Levin B, Ajani JA, Faintuch JS, Saks S, Patt YZ, *et al*. Phase I trial of recombinant human g-interferon and recombinant human tumor necrosis factor in patients with advanced gastrointestinal cancer. *Cancer Res* 1989; **49**:4057–4061.
13 Blick M, Sherwin SA, Rosenblum M, Gutterman J. Phase I study of recombinant tumor necrosis factor in cancer patients. *Cancer Res* 1987; **47**:2986–2989.
14 Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, *et al*. NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. *Proc Natl Acad Sci U S A* 1990; **87**:3629–3632.
15 Van der Poll T, van Deventer SJ, Hack CE, Wolbink GJ, Aarden LA, Büller HR, *et al*. Effects on leukocytes after injection of tumor necrosis factor into healthy humans. *Blood* 1992; **79**:693–698.
16 Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, *et al*. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNF-dependent apoptosis. *Cell* 2007; **131**:669–681.
17 Gerspach J, Németh J, Münkel S, Wajant H, Pfizenmaier K. Target-selective activation of a TNF prodrug by urokinase-type plasminogen activator (uPA) mediated proteolytic processing at the cell surface. *Cancer Immunol Immunother* 2006; **55**:1590–1600.
18 Cherix S, Speiser M, Matter M, Raffoul W, Liénard D, Theumann N, *et al*. Isolated limb perfusion with tumor necrosis factor and melphalan for non-resectable soft tissue sarcomas: long-term results on efficacy and limb salvage in a seleted group of patie. *J Surg Oncol* 2008; **98**:148–155.
19 Bellavance EC, Alexander HR Jr. TNF-based isolated hepatic perfusion. *Front Biosci* 2009; **14**:1771–1784.
20 Wang H, Chen K, Cai W, Li Z, He L, Kashefi A, *et al*. Integrin-targeted imaging and therapy with RGD4C-TNF fusion protein. *Mol Cancer Ther* 2008; **7**:1044–1053.
21 Nakamura S, Kato A, Masegi T, Fukuoka M, Kitai K, Ogawa H, *et al*. A novel recombinant tumor necrosis factor-a mutant with increased anti-tumor activity and lower toxicity. *Int J Cancer* 1991; **48**:744–748.
22 Van Ostade X, Vandenabeele P, Everaerdt B. Human TNF mutants with selective activity on the p55 receptor. *Nature* 1993; **361**:266–269.
23 Shin NK, Lee I, Chang SG, Shin HC. A novel tumor necrosis factor-alpha mutant with significantly enhanced cytotoxicity and receptor binding affinity. *Biochem Mol Biol Int* 1998; **44**:1075–1082.
24 Kuroda K, Miyata K, Fujita F, Koike M, Fujita M, Nomura M, *et al*. Human tumor necrosis factor-alpha mutant RGD-V29 (F4614) shows potent antitumor activity and reduced toxicity against human tumor xenografted nude mice. *Cancer Lett* 2000; **159**:33–41.
25 Creasey AA, Doyle LV, Reynolds MT, Jung T, Lin LS, Vitt CR. Biological effects of recombinant human tumor necrosis factor and its novel muteins on tumor and normal cell lines. *Cancer Res* 1987; **47**:145–149.
26 Yan Z, Zhao N, Wang Z, Li B, Bao C, Shi J, *et al*. A mutated human tumor necrosis factor-alpha improves the therapeutic index in vitro and in vivo. *Cytotherapy* 2006; **8**:415–423.
27 Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA Jr, Goeddel DV. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* 1991; **88**:9292–9296.
28 Barbara JA, Smith WB, Gamble JR, Van Ostade X, Vandenabeele P, Tavernier J, *et al*. Dissociation of TNF-alpha cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-alpha mutants. *EMBO J* 1994; **13**:843–850.
29 Van Zee KJ, Stackpole SA, Montegut WJ, Rogy MA, Calvano SE, Hsu KC, *et al*. A human tumor necrosis factor (TNF) alpha mutant that binds exclusively to the p55 TNF receptor produces toxicity in the baboon. *J Exp Med* 1994; **179**:1185–1191.
30 Li X, Yang Y, Ashwell JD. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature* 2002; **416**:345–347.
31 Bouwmeester T, Bauch A, Ruffner H, Angrand PO, Bergamini G, Croughton K, *et al*. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat Cell Biol* 2004; **6**:97–105.
32 Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* 1996; **274**:787–789.
33 Heller RA, Song K, Fan N, Chang DJ. The p70 tumor necrosis factor receptor mediates cytotoxicity. *Cell* 1992; **70**:47–56.
34 Depuydt B, Van Loo G, Vandenabeele P, Declercq W. Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors. *J Cell Sci* 2005; **118**:497–504.
35 Baldwin RL, Mirzabekov T, Kagan BL, Wisnieski BJ. Conformation and ion channel activity of lymphotoxin at neutral and low pH. *J Immunol* 1995; **154**:790–798.
36 Soma G, Kitahara N, Tsuji Y, Kato M, Oshima H, Gatanaga T, *et al*. Improvement of cytotoxicity of tumor necrosis factor (TNF) by increase in basicity of its N-terminal region. *Biochem Biophys Res Commun* 1987; **148**:629–635.
37 Kamijo R, Takeda K, Nagumo M, Konno K, Hasegawa A, Inaka K, *et al*. Induction of differentiation of human monoblastic and myeloblastic leukemia cell lines by TNF muteins. *Biochem Biophys Res Commun* 1989; **160**:820–827.
38 Meng JR, Zhao N, Yan Z, Li YS, Wang WY, Chang YM, *et al*. Expression of TNFRs in tumor cells and effect of novel rmhTNF on tumor cell growth. *Chin J Cell Mol Immunol Chinese* 2002; **18**:10–13.
39 Elia N, Tapponnier M, Matthay MA, Hamacher J, Pache JC, Brundler MA, *et al*. Functional identification of the alveolar edema reabsorption activity of murine tumor necrosis factor-alpha. *Am J Respir Crit Care Med* 2003; **168**:1043–1050.
40 Wang H, Yan Z, Shi J, Han W, Zhang Y. Expression, purification, and characterization of a neovasculature targeted rmhTNF-alpha in Escherichia coli. *Protein Exp Purif* 2006; **45**:60–65.