# A novel human tumor necrosis factor α mutant showed potent antitumor activity and reduced toxicity *in vivo*[1]

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**KEY WORDS** tumor necrosis factor; experimental implants; antineoplastic agents; toxicity tests; nude mice; macaca mulatta

## ABSTRACT

**AIM**: To study the antitumor activity and systemic toxicity of human tumor necrosis factor α (hTNFα) mutant M2 (R2K-N30S-R32W-L157F-hTNFα). **METHODS**: Mouse sarcoma S180 tumors and hepatoma HAC tumors were implanted into mice, and human urocyst carcinoma CP-3 tumors were implanted into nude mice. The xenografted mice were injected with wild-type hTNFα and its mutant M2 at different doses. After 7 d (mice) or 10 d (nude mice) of injection, the tumor weight was measured to calculate the inhibition rate of hTNFα and M2. Systemic toxicity experiments were done on Rhesus monkeys by injecting them with wild-type hTNFα and mutant M2 respectively for 10 consecutive d. Observations were made on the monkeys both before and after the injection. **RESULTS**: For mice implanted with sarcoma S180 and hepatoma HAC tumors, the inhibition rate of M2 was similar to that of wild-type hTNFα at the dose of 0.025 mg/kg, while for nude mice implanted with human urocyst carcinoma CP-3, the inhibition rate of M2 (45.5 %) was much higher than that of wild-type hTNFα (15.5 %). When the dose came to 0.25 and 2.5 mg/kg respectively, however, the inhibition rate of M2 greatly increased (the highest was 75.9 %). The tests of systemic toxicity of hTNFα and its mutant M2 in monkeys proved that M2 presented lower toxicity than wild-type hTNFα did. **CONCLUSION**: hTNFα mutant M2 not only presented higher antitumor activity than wild-type hTNFα did on mouse tumor (S180 and HAC)- and human tumor (CP-3)-implanted mice, but also showed lower systemic toxicity in the Rhesus monkey.

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## INTRODUCTION

Human tumor necrosis factor α (hTNFα) is a cytokine primarily produced by activated macrophages and first discovered by its ability to cause hemorrhagic necrosis of tumors *in vivo* as well as its cytotoxic activity on malignant cells *in vitro*[1]. However, administration of murine TNF as an antitumor agent revealed a high *in vivo* toxicity in mice[2-4] and injection of high doses of hTNF in humans, which was necessary for tumor regression, caused serious side effects, such as hypotension and hepatotoxicity[1]. This toxicity most probably reflected the pleiotropic character of TNF and its important role in inflammatory and immune responses.

To use TNFα as an anticancer drug, the structure-function relationship of hTNFα was investigated and many hTNFα mutants were reported. van Ostade *et al*[5] found that compared with wild-type hTNFα, mutant R32W-hTNFα showed similar cytotoxicity to HEp-2 cells while its inflammatory effects *in vitro* were lower than wild-type hTNFα. Moreover, the *in vivo* antitumor activity of R32W in xenografted nude mice was similar to that of wild-type hTNFα. Based on the studies of R32W-hTNFα and the other mutants, we established a novel TNFα combined mutant M2 (R2K-N30S-R32W-L157F-hTNFα) which had a lower cytotoxicity to L929 cells (murine fibroblasts) and high cytotoxic activities to several kind of human tumor cells, such as HEp-2, MCF-7, HepG-2 and 3AO[6]. Furthermore, the mouse acute toxicity experiment showed that the $LD_{50}$ of hTNFα mutant M2 was at least 700 fold lower than the $LD_{50}$ of wild-type hTNFα[6]. In the present study, to evaluate the antitumor activity of M2 *in vivo*, the antitumor effect of the mutant was compared with wild-type TNFα in xenografted

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mice and nude mice, and systemic toxicity of M2 in monkeys was also evaluated.

## MATERIALS AND METHODS

**Reagents and cytokines** Cyclophosphamide (CTX) was from Shanghai Hualian Pharmaceutical Co Ltd (China). The kits for serum biochemistry examination were from Beijing Zhongsheng Biotechnology Hi-tech Company (China). Wild type TNFα (8.3 g/L) and mutant M2 (12.8 g/L) were expressed in *E coli* and purified in our lab with the purity of about 95 %.

**Tumor lines** Mouse tumor sarcoma S180 and hepatoma HAC were from Shanghai Institute of Pharmaceutical Industry (China); human urocyst carcinoma CP-3 was from Shanghai Experimental Animal Center of Chinese Academy of Sciences.

**Animals** Male BALB/c nude mice, weighing (19.43±0.22) g (Grade Ⅱ, Certificate № 005, from Shanghai Experimental Animal Center, Chinese Academy of Sciences, China), male Kunming strains mice, weighing (19.44 ± 0.19) g (Grade Ⅱ, Certificate № 107, from Shanghai Institute of Pharmaceutical Industry, China), and Rhesus monkeys, 5-year-old, of both sexes, weighing (5.6 ± 0.6) kg (Certificate № BDW95002, from Experimental Animal Center, Academy of Military Medical Sciences, Beijing, China) were housed individually and fed three times a day with free access to water.

**Antitumor activity assay**[7] Physiological saline was added to tumor tissues at a ratio of 4:1, followed by homogenization. After mice were administered with hypodermic injection of the tumor cell suspension (0.2 mL/mouse), they were randomly divided into groups (10 mice per group): physiological saline (normal saline) group as a negative control; CTX group as a positive control; wild-type hTNFα group (0.025 mg/kg) and M2 group which was subdivided into three groups of 0.025 mg/kg, 0.25 mg/kg, and 2.5 mg/kg respectively. The xenografted mice were injected with hTNFα for continuously 7 d (0.5 mL/mouse). After 10 d implantation, mice were dissected and the tumors were taken out to compare their weight. The xenografted nude mice were injected with hTNFα for continuously 10 d (0.5 mL/mouse). After 15 d implantation, the nude mice were dissected and the tumors were taken out to compare their weight. The rate of antitumor activity was calculated as:

Inhibition rate (%) = (Average tumor weight of control group − Average tumor weight of test group)/Average tumor weight of control group × 100 %

**Toxicity test** Rhesus monkeys were divided into two groups: wild-type hTNFα group and M2 group (4 monkeys per group). Monkeys were administered intravenously with hTNFα (200 μg/m² body surface area) on 10 consecutive d. The activity, behavior, food-taking, and death of the animals were observed. The monkeys were weighed and given blood tests both before and after injection. The serum biochemistry examination included total protein (TP), albumin (ALB), glucose (GLU), total bilirubin (T-BIL), creatinine (Crea), total cholesterol (T-CHO), blood urea nitrogen (BUN), glutamic pyruvic transaminase (ALT), glutamic oxaloacetic transaminase (AST), and alkaline phosphatase (ALP).

**Data analysis** The data are presented as $\bar{x} \pm s$. The *t*-test was used to estimate the statistical significance.

## RESULTS

**Antitumor activity of M2 in xenografted mice and nude mice** For mouse sarcoma S180, at the dose of 0.025 mg/kg, the inhibition rate of hTNFα mutant M2 was higher than that of wild-type (53.2 % and 30.2 % respectively). When the dose of M2 came to 0.25 and 2.5 mg/kg, the inhibition rate reached 59.4 % and 69.1 % (Tab 1). For mouse hepatoma HAC, the inhibition rate of wild-type hTNFα (0.025 mg/kg) was 45.5 % while that of M2 was 38.7 %, 62.4 % and 75.9 % at the dose of 0.025, 0.25, and 2.5 mg/kg respectively (Tab 1).

**Tab 1. Antitumor activity of wild-type hTNFα and its mutant M2 on mouse sarcoma S180 and hepatoma HAC. *n* = 10 mice. $\bar{x} \pm s$. ᶜ$P < 0.01$ *vs* normal saline group.**

| | Groups | Dose/mg·kg⁻¹ | Tumor weight/g | Inhibition rate/% |
|---|---|---|---|---|
| S180 | Normal saline | 10 mL/kg | 2.8±0.5 | – |
| | CTX | 100 | 0.13±0.05ᶜ | 95.3 |
| | Wild-type hTNFα | 0.025 | 1.9±0.6ᶜ | 30.6 |
| | Mutant M2 | 0.025 | 1.3±0.4ᶜ | 53.2 |
| | | 0.25 | 1.13±0.21ᶜ | 59.4 |
| | | 2.5 | 0.86±0.25ᶜ | 69.1 |
| HAC | Normal saline | 10 mL/kg | 2.7±0.6 | – |
| | CTX | 100 | 0.14±0.05ᶜ | 94.7 |
| | Wild-type hTNFα | 0.025 | 1.5±0.5ᶜ | 45.5 |
| | Mutant M2 | 0.025 | 1.6±0.5ᶜ | 38.7 |
| | | 0.25 | 1.0±0.5ᶜ | 62.4 |
| | | 2.5 | 0.64±0.21ᶜ | 75.9 |

M2 also presented high antitumor activity to human urocyst carcinoma CP-3 in nude mice. The inhibition rate of wild-type hTNFα was 15.5 % at the dose of 0.025 mg/kg while that of M2 was 45.5 %, 52.1 %, and 64.1 % at the dose of 0.025, 0.25, and 2.5 mg/kg, respectively (Tab 2). It was clear that M2 presented much higher antitumor activity to human CP-3 tumors than wild-type hTNFα did.

**Tab 2. *Antitumor activity of wild-type hTNFα and mutant* M2 to human urocyst carcinoma in nude mice. $n = 10$ mice. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normal saline group.**

| Groups | Dose/ $mg \cdot kg^{-1}$ | Tumor weight/g | Inhibition rate/% |
|---|---|---|---|
| Normal saline | 10 mL/kg | 1.67±0.26 | – |
| CTX | 100 | 0.080±0.021$^c$ | 95.2 |
| Wild-type hTNFα | 0.025 | 1.4±0.7$^c$ | 15.5 |
| Mutant M2 | 0.025 | 0.9±0.4$^c$ | 45.5 |
| | 0.25 | 0.80±0.27$^c$ | 52.1 |
| | 2.5 | 0.6±0.3$^c$ | 64.1 |

**Toxicity of hTNFα mutant M2 in monkeys**

Two monkeys of the wild-type hTNFα group died in 10 d, and another monkey died after the 10 d injection. Meanwhile, the monkeys of M2 group all survived with normal activities after the 10 d injection.

In the M2 group, the weight of monkeys slightly decreased after the 10 d injection (Tab 3). However, the decrease of average weight was not much different ($P > 0.05$). In the blood tests (Tab 4), all the serum biochemistry indexes except ALP were normal ($P > 0.05$) after injecting M2 ($200\ \mu g \cdot m^{-2} \cdot d^{-1}$, 10 d).

**Tab 3. Effect of mutant M2 ($200\ \mu g \cdot m^{-2} \cdot d^{-1}$, iv, for 10 d) on body weight of monkeys.**

| Weight/kg | 1 | 2 | 3 | 4 | $\bar{x} \pm s$ | $P$ |
|---|---|---|---|---|---|---|
| Before injection | 6.3 | 5.5 | 5.9 | 4.8 | 5.6±0.6 | >0.05 |
| After injection | 5.8 | 5.4 | 5.4 | 4.1 | 5.2±0.7 | >0.05 |

**Tab 4. Effects of mutant M2 ($200\ \mu g \cdot m^{-2} \cdot d^{-1}$, iv, for 10d) on the blood biochemistry indexes of monkeys. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* before injection.**

| Blood biochemistry Indexes | Unit | Before injection | After injection |
|---|---|---|---|
| ALT | U/L | 55±19 | 25±8$^b$ |
| AST | U/L | 74±26 | 61±17 |
| ALP | U/L | 105±44 | 135±50 |
| BUN | mmol/L | 8.5±2.2 | 9.5±1.4 |
| Crea | μmol/L | 93±17 | 101±14 |
| TP | g/L | 87±8 | 76.8±7 |
| ALB | g/L | 50±5 | 43±6 |
| GLU | mmol/L | 3.9±0.7 | 4.6±0.8 |
| T-CHO | mmol/L | 3.6±0.7 | 3.8±1.1 |
| T-BIL | μmol/L | 7.0±2.0 | 5.0±2.2 |

## DISCUSSION

We had expressed some novel hTNFα mutants in *E coli* and studied their cytotoxicity, receptor binding activities and systemic toxicity ($LD_{50}$ in mice)[6]. hTNFα mutant M2 presented high cytotoxicity to several human tumor cell lines but showed very low cytotoxicity to L929 cells[6]. Moreover, the mutant hTNFα had much lower systemic toxicity in mice than wild-type did[6]. Loetscher *et al* found that mutant R32W bound to murine TNF receptor with affinities about 1000-fold lower than that of wild-type hTNFα[8]. So a question was raised as whether M2 really had low toxicity or it was only unable to affect mice.

In the present study, we first revealed the curative effect of hTNFα mutant M2 *in vivo*. At the dose of 0.025 mg/kg, M2 had similar antitumor activity with that of wild-type hTNFα to mouse S180 and HAC tumors in mice. Because the systemic toxicity of M2 in mice was much lower than that of Wild-type hTNFα, the xenografted mice could be safely injected with higher concentration (0.25 and 2.5 mg/kg) which was lethal dose for wild-type hTNFα. The present results showed that at the dose of 2.5 mg/kg the inhibition rate of M2 was as high as 65 % – 75 %, a level that wild-type hTNFα couldn't reach. It suggested that M2 exerted better curative effect at higher dose that was safe for mice.

Moreover, The curative effect experiments of M2 on human tumors further demonstrated the potential of the hTNFα mutant in clinical trails. In our studies with human urocyst carcinoma CP-3, M2 presented much higher antitumor activity than wild-type hTNFα did at the same dose, with the inhibition rate of 45.5 % and 15.5 % respectively.

Besides R32W-hTNFα, several novel hTNFα mutants which had potent antitumor activity with reduced toxicity in mice were reported. As Kuroda *et al* described[9], hTNF mutant V29 ($^1$SSS$^4$RTPSDK ...$^{29}$VR...$^{155}$L) had potent antitumor activity with reduced toxicity in mice while another mutant RGD-T

([1]SSS[4]RGDSDK...[29]RR...[155]L) showed decreased gastrointestinal toxicity. The recently reported combined mutant RGD-V29 ([1]SSS[4]RGDSDK...[29] VR...[155] L) showed no hypotensive effects in rats[10], but possessed more potent antitumor effects than wild-type hTNFα in syngeneic Meth A-bearing mice and heterogeneic human lung cancer Mqnu-1-bearing mice[11].

Because human TNF does not bind to the mouse p75 receptor, $LD_{50}$ in mice is not enough to evaluate all TNF toxicities to human. Thus, we examined the toxicities of TNF in monkeys. TNF can induce anorexia, thermogenesis, and fat and protein mobilization and each of these factors contributes to the loss of body weight[1]. The involvement of TNF in loss of body weight was reported in the experiments that animals were injected or infused with TNF[12,13]. It seemed that hTNFα mutant M2 didn't present the obvious side effect as wild-type hTNFα by observing its effect to body weight of the monkey. The blood tests also showed that M2 had no effect to the serum biochemistry index and proved its low systemic toxicity. Thus, M2 appears to be a low-toxicity mutant of rhTNF that shows preferential activity towards tumors, and therefore merits further investigation in pre-clinical and clinical studies.

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## 一种新型人肿瘤坏死因子 α 突变体具有高抑瘤活性及低体内毒性[1]

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**关键词** 肿瘤坏死因子; 实验性植入物; 抗肿瘤药; 毒性试验; 裸小鼠; 猕猴

**目的**: 研究人肿瘤坏死因子 α (hTNFα)新型突变体 M2(R2K-N30S-R32W-L157F-hTNFα)的动物体内抑瘤效应及毒性. **方法**: 将小鼠肉瘤 S180 及肝癌 HAC 实体瘤细胞分别植入小鼠, 并将人膀胱癌瘤株 CP-3 植入裸鼠体内, 再对它们分别注射不同剂量的野生型 hTNFα 以及突变体 M2. 连续注射 7d (小鼠)或 10 d (裸鼠)后称实体瘤重量, 据此计算野生型 hTNFα和 M2 的抑瘤率. 对恒河猴进行为期 10 d 的多次注射野生型 hTNFα 及其突变体 M2 的毒性实验, 并观察注射前后动物的生理状况, 检查体重及血液生化指标. **结果**: 对于小鼠移植瘤 S180 和 HAC, 在 0.025 mg/kg 的给药浓度下, 突变体 M2 表现出与野生型 hTNFα 相似的抑瘤效应, 而在这一浓度下, M2

对移植到裸鼠的人膀胱癌 CP-3 肿瘤的抑瘤率为 45.5 %，明显高于野生型 hTNFα 的 15.5 %．此外，当 M2 的给药浓度提高到 0.25 及 2.5 mg/kg 时，其抑瘤率明显升高，其中最高可达 75.9 %．恒河猴体内毒性实验结果证明，hTNFα 突变体 M2 的毒性要小于野生型 hTNFα．**结论**：hTNFα 新型突变体 M2 相对于野生型 hTNFα 不仅在小鼠肿瘤（S180 和 HAC）和人肿瘤（CP-3）移植的小鼠中具有更高的抑瘤活性，而且在恒河猴体内表现较低的毒性．

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