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Humanization of Murine Monoclonal anti-hTNF Antibody: The F10 Story[1]

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Abstract—Tumor necrosis factor (TNF) is a proinflammatory cytokine implicated in pathogenesis of multiple autoimmune and inflammatory diseases. Anti-TNF therapy has revolutionized the therapeutic paradigms of autoimmune diseases and became one of the most successful examples of the clinical use of monoclonal antibodies. Currently, anti-TNF therapy is used by millions of patients worldwide. At the moment, fully human anti-TNF antibody Adalimumab is the best-selling anti-cytokine drug in the world. Here, we present a story about a highly potent anti-TNF monoclonal antibody initially characterized more than 20 years ago and further developed into chimeric and humanized versions. We present comparative analysis of this antibody with Infliximab and Adalimumab.

Keywords: TNF, tumor necrosis factor, recombinant antibodies, humanized antibodies
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INTRODUCTION

Tumor necrosis factor (TNF) is a proinflammatory cytokine, which under normal conditions mediates important functions in lymphoid system development and host defense. The pathological role of TNF overexpression in various autoimmune diseases was discovered in the 1980s and very soon the idea that its inhibition may be beneficial for autoimmune patients took hold (for review see [1, 2]. Indeed, anti-TNF therapy became a major success demonstrating that inhibition of a single cytokine can disrupt the inflammatory vicious circle. TNF-specific monoclonal antibodies or engineered soluble receptors proved to be effective in patients and became an important therapeutic option for numerous disorders of autoimmune and inflammatory nature: rheumatoid arthritis, ankylosing spondylitis, Crohn's disease and psoriasis.

F10 is a murine monoclonal antibody, specific for human TNF and possessing very high avidity and inhibitory potency. F10 was associated with a rather sad story briefly outlined below. F10 hybridoma was generated in 1987 in Minsk, in the laboratory of N.N. Voitenok by a talented young scientist, A.V. Panyutich (who untimely passed away in 2002). A. Panyutich developed an immunization protocol that utilized very high doses of human TNF. As it turned out later, surviving mice developed antibodies with very high neutralizing activity. Some of his antibodies were later characterized by Petyovka et al. [3], but F10 remained unpublished at that time. Recombinant human TNF for these studies was expressed and purified in the late 1980s in the laboratory of V.G. Korobko (who passed away in 2001) based on earlier cloning work [4]. Then another young scientist, B.V. Radko, has cloned light and heavy chains from the F10 hybridoma and determined their nucleotide sequences [5]. He untimely passed away in 2007.

We later used these published sequences to construct a recombinant single-chain antibody (scFv) that possessed similar inhibitory activity as the Fab fragment of the original F10 antibody [6], demonstrating that these VH and VL genes could be used for further antibody engineering.

Around that time a novel mouse strain was developed in our laboratory, in which the murine *tnf* gene was replaced by homologous recombination with its human counterpart [7, 8]. In these mice, designated as hTNF KI (human TNF knock-in), both protective and pathogenic functions are mediated by human TNF, making this system an ideal model for evaluating efficacy of hTNF inhibitors in vivo. Furthermore, pri-

[1] The text was submitted by the author(s) in English.

mary cells from the same mice can be used for various experiments in vitro.

We describe below an experimental study with chimeric and humanized variants of F10, in which we compared their properties with well-established and clinically used TNF-inhibitors Infliximab and Adalimumab.

EXPERIMENTAL

Generation of chimeric anti-TNF antibody. To generate a chimeric full-length anti-TNF antibody (chF10), we have cloned genes encoding the VH and VL domains of the F10 antibody into mammalian expression vectors, containing the human IgG1 (allotype G1m17.1.) and IgK constant domains, respectively.

Generating humanized antibodies. For generating the humanized versions of the anti-TNF molecule, based on the published sequences of anti-TNF V_H and V_L [5], first we performed the search in IMGT human database of germline sequences for the closest human germline VH and VL sequences. VH IGHV7-4-1*02, and VKappa IGKV1-6*01 were the best aligned sequences.

Subsequently, we used the Yasara software package [9] to perform homology modeling of the original F10 mouse clone. Individual residues from the mouse VH en VKappa, residing outside the CDRs, which were suspected to influence folding of the loops were back-mutated to the mouse residues into the original humanized chains in various combinations, resulting in 3 VH and 4 VKappa chains. These genes were cloned into mammalian expression vectors containing the human IgG1 allotype G1m17.1 and IgK constant domains, respectively. All possible combinations of the V_H and V_L chains were expressed in HEK293 cells. All twelve clones were tested for affinity and we finally ended up with two clones with a very low percentage of mouse residues, clones #13242 and #13245, and high affinity. These were expressed in HEK293 cells at a larger scale (1 liter) to allow further experimentation.

Antibody purification. Large scale antibody productions were purified by protein A affinity chromatography. In short, supernatants were harvested and subsequently clarified and concentrated by using a KrosFlo research IIi TFF system (Spectrum Labs). All antibodies were purified from cell-free supernatant using protein A (MabSelect SuRe; GE Healthcare) affinity chromatography on an ÄKTA-FPLC machine (GE Healthcare). Antibodies were eluted with 100 mM sodium citrate-HCl, pH 3.0 and immediately neutralized with 1 M Tris, pH 9.0. Next, antibodies were desalted with the ÄKTA-FPLC and HiPrep 26/10 columns (GE Healthcare) to PBS, pH 7.4. After 0.2 μm filtration, antibody concentrations were determined by absorbance at 280 nm and a mass coefficient of 13.5 for a 1% (10 mg/mL) IgG solution (NanoDrop; Thermo Scientific).

Cytotoxic assay. TNF sensitive murine fibrosarcoma cells L929 or WEHI 164 Clone 13 [10] were plated in 96-well culture plates (5000 cells/well for L929 or 20000 cells/well for WEHI 164. Recombinant human TNF was added at a concentration of 100 U/mL (for L929 cell line media was supplemented also with and Actinomycin-D (Sigma-Aldrich, A5156) at 4 μg/mL). TNF-inhibitors were applied at serial dilutions from 50 nM—3 pM. After 24 h incubation Methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, M5655) was added to a final concentration of 1 mM. After 4 h formazan crystals were solubilized by 10% w/v SDS in DMSO and optical density was measured at 540 nm with a 492 nm reference. Percentage of living cells was calculated and fitted to nonlinear regression curve using GraphPad Prism software.

LPS/D-Galactosamine-induced acute hepatotoxicity model. hTNF KI mice aged 6–8 weeks were randomly distributed in groups (4/5 mice per group) with equal ratio of male and female mice in each group. HuAb and Adalimumab were injected i.p. each at 75, 25, 10 or 3 pmol per gram of mouse weight. The control group received vehicle buffer only (PBS). 30 min later mice were injected i.p. with 400 ng/g LPS (Sigma-Aldrich, L2630) and 800 μg/g D-Galactosamine (Sigma-Aldrich, G1639). Mice were observed for 2000 min following LPS/D-Gal injection. Time of death was noted for each animal. Kaplan-Meier survival curves were plotted for each concentration using Prism software (GraphPad).

Measurement of antibody affinity by surface plasmon resonance. SPR experiments were performed using the ProteOn XPR36 system (Bio-Rad). Phosphate buffered saline with 0.005% Tween 20, pH 7.4 was used as running buffer throughout, kinetic was measured at 25°C. Recombinant hTNF was expressed in *E. coli* and purified as described previously [11].

According to previously published protocol [12], first a goat polyclonal anti-human IgG were immobilized on a ProteOn GLC sensor chip (Bio-Rad) using standard amine coupling chemistry. Next, TNF antagonists were immobilized in the same direction. Then, five analyte concentrations in two-fold dilutions (hTNF 50–3 nM) were injected into six analyte channels orthogonal to the ligand channels. Thus, all hTNF dilutions reacted simultaneously with different TNF inhibitors in a single injection. Running buffer was injected into the sixth analyte channel, which was used as a reference. The data were analyzed and fitted to a 1 : 1 Langmuir interaction model by ProteOn Manager™ software (Bio-Rad). At least three independent experiments were performed.

RESULTS AND DISCUSSION

Chimeric anti-TNF Antibody

Chimeric anti-TNF antibody (chF10) was expressed in mammalian cells, purified by affinity chromatogra-

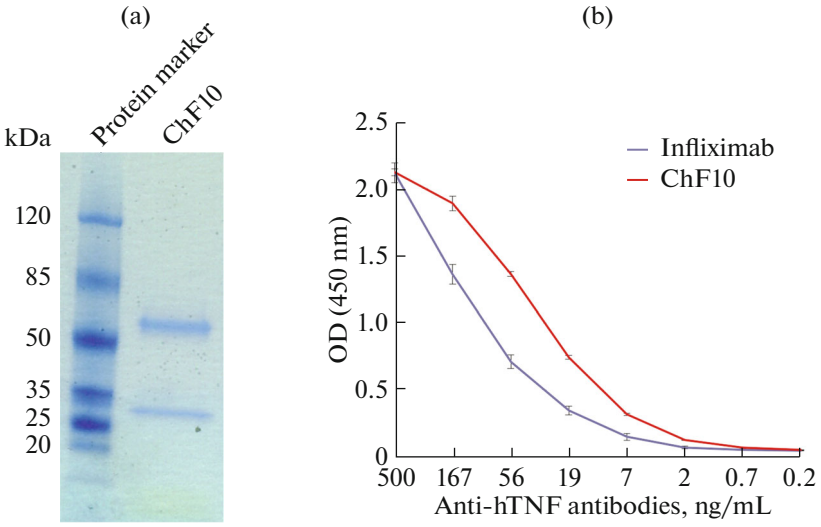

Fig. 1. Analysis of chimeric anti-TNF Antibody. (a) SDS electrophoresis. Heavy and light chains of antibody are visible (molecular weights of protein markers are indicated). (b) ELISA of binding of chimeric anti-TNF antibody (ChF10) compared to Infliximab.

phy (Fig. 1a) its binding to human TNF was confirmed by ELISA (Fig. 1b).

To directly measure and compare affinity of F10 and chimeric F10 (ChF10) to Infliximab we employed surface plasmon resonance technology using the previously offered approach [12]. It utilizes secondary anti-human IgG antibody to coat interaction chip surface and then TNF inhibitors are non-covalently attached to it. Thus, all antibodies were immobilized in the correct orientation leaving variable domain accessible for interaction with TNF.

We have demonstrated that ChF10 have superior affinity when compared to Infliximab (Fig. 2).

To test if higher affinity of F10 and ChF10 results in increased activity in inhibiting TNF biological effects we have utilized the cytotoxic assay on L929 cell line. Indeed, F10 and ChF10 appeared more potent than Infliximab (Fig. 3).

Fully Humanized anti-TNF mAbs

With the use of CDR grafting technique we have generated two high-affinity fully humanized anti-TNF antibodies #13242 and #13245. Both antibodies were produced in HEK293 cells, purified by protein A affinity chromatography and desalted.

We have compared affinity of two clones of HuAb to Infliximab by the use of surface plasmon resonance. Both Abs had higher calculated affinity than Infliximab (Fig. 4).

Next we proceeded to cytotoxic test. As with ChF10 we have tested HuAb in cytotoxic assay on L929 cells and demonstrated that clone #13242 was superior to Infliximab and almost as active as chimeric and original F10 antibody while clone #13245 was less active in TNF inhibition assay (Fig. 5a). For clone #13242 its increased TNF-inhibition activity corresponded to superior affinity measured by surface plasmon resonance. Moreover we have compared two clones of humanized antibodies to two other commercially available TNF inhibitors Adalimumab and Golimumab, which are fully human antibodies and confirmed that clone #13242 was as active as chimeric antibody and superior to both Adalimumab and Golimumab (Fig. 5b). In a separate experiment on another cell culture (Wehi 164 Cl. 13) we have confirmed that humanized antibody have more inhibitory activity than Adalimumab, the most potent of all clinically used anti-TNF antibodies (Fig. 5c).

To test the TNF-inhibition potency of developed anti-TNF HuAb in vivo and compare it to the most widely used anti-TNF human antibody Adalimumab, we utilized the acute hepatotoxicity model in hTNF KI mice [7, 8]. Mice were injected with 75, 25 or 10 pmol/g of TNF inhibitors 30 before lethal dose of LPS and D-galactosamine. HuAb as well as Adalimumab protected the mice from toxicity (Fig. 6a) (Mean ± SD and nonlinear regression of dose-dependent inhibition of TNF mediated cytotoxicity are shown).

(a) To check if increased activity of HuAb in cytotoxic test corresponds to higher in vivo potency, we have titrated TNF-inhibitors even further and found that it indeed protected the mice at a dose as low as 3 pmol/g (450 ng/g) when Adalimumab was no longer efficient (Fig. 6b) (Mean ± SD and nonlinear regression of dose-dependent inhibition of TNF mediated cytotoxicity are shown).

(b) This confirmed that HuAb is more effective at TNF inhibition, which probably is explained by its increased affinity.

We described here chimeric and humanized versions of an original anti-TNF monoclonal antibody

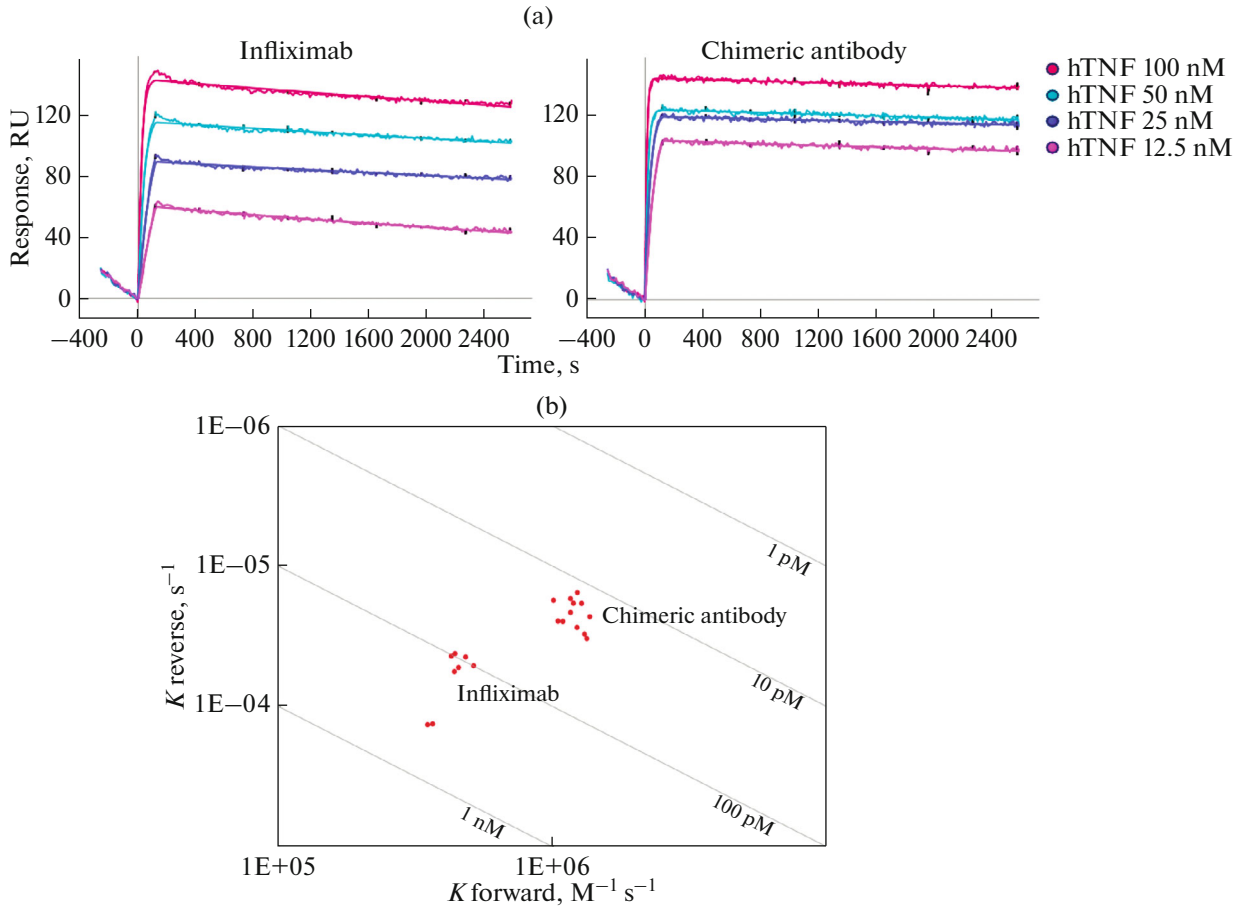

Fig. 2. Affinity measurement of binding of chimeric anti-TNF antibody compared to Infliximab. (a) Representative interaction curves of chimeric anti-TNF antibody and Infliximab at different TNF concentrations. Recombinant human TNF concentrations are indicated. (b) Isoaffinity graph. Each dot represents an independent measurement.

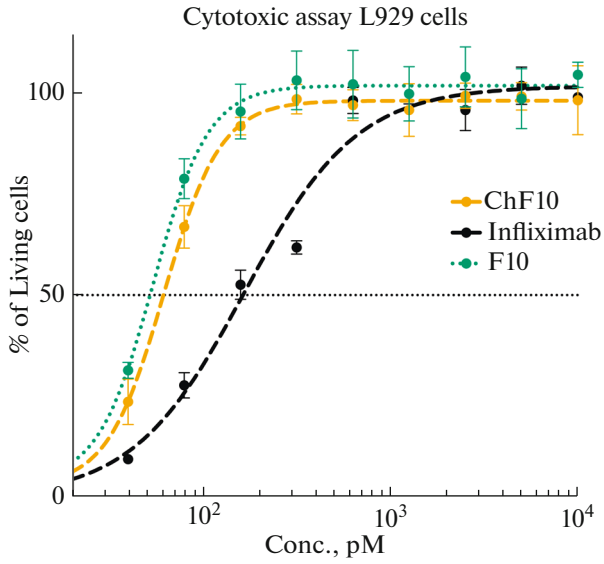

Fig. 3. TNF inhibition assay on L929 cell. Mean ± SD and nonlinear regression of dose-dependent inhibition of TNF mediated cytotoxicity are shown.

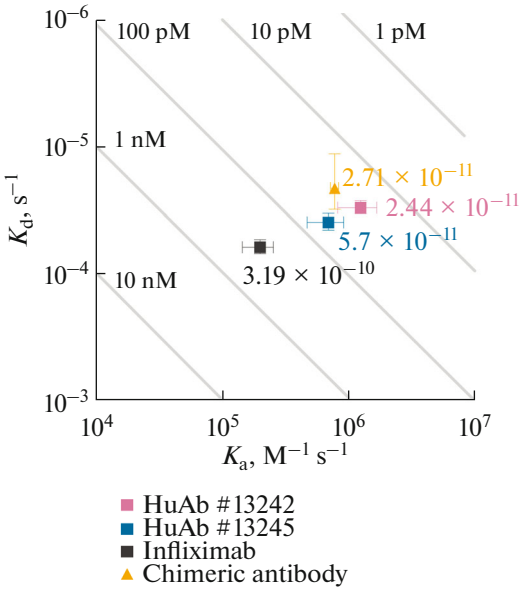

Fig. 4. Affinity of interaction of two clones of humanized anti-TNF antibodies compared to Infliximab. Isoaffinity graph mean values ± SD are shown.

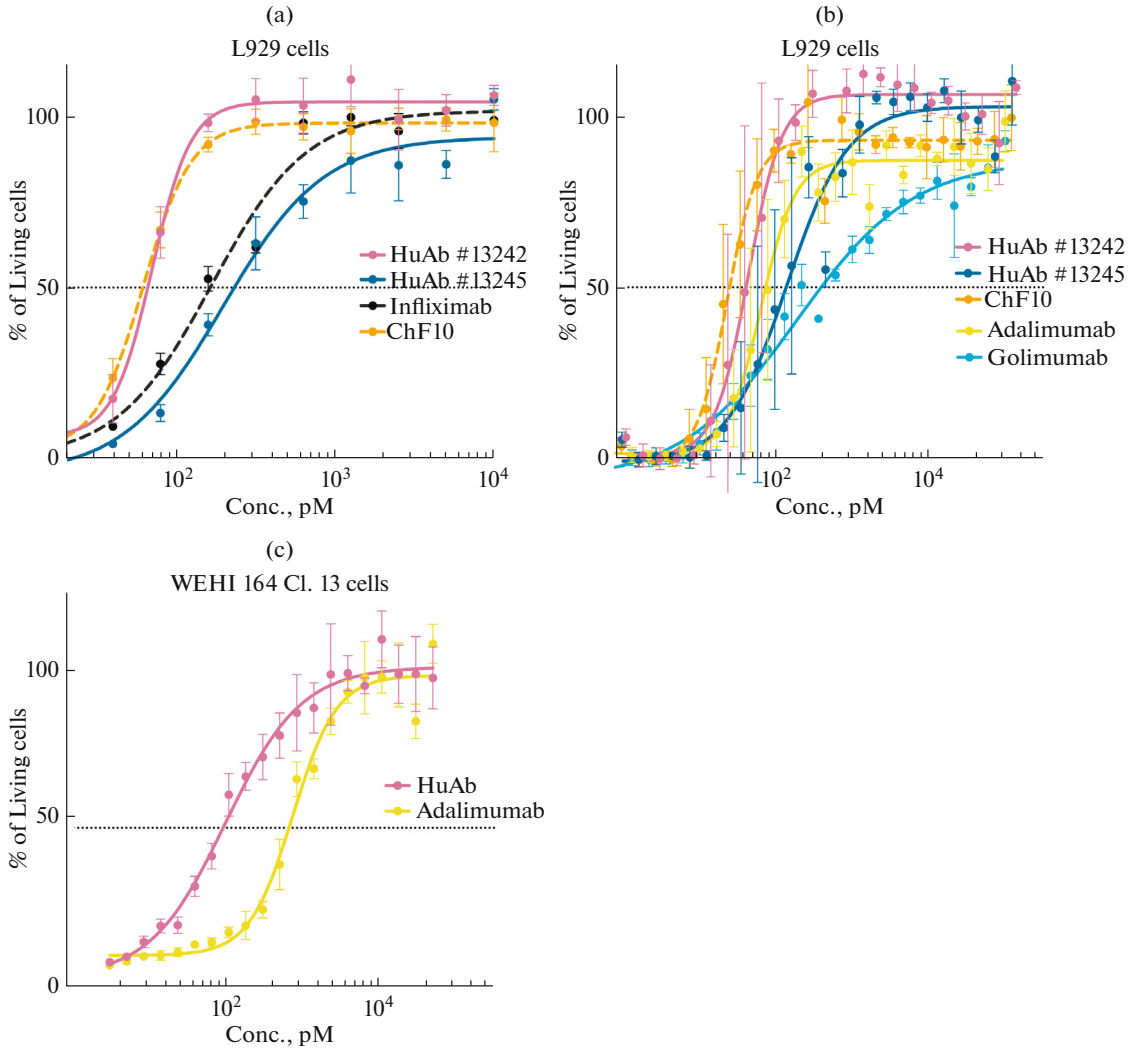

Fig. 5. TNF inhibition assay. (a) 2 clones of anti-TNF HuAb and chimeric antibody (ChF10) compared to Infliximab (L9292 cells). (b) 2 clones of anti-TNF HumAb and chimeric antibody (ChF10) compared to Adalimumab and Golimumab (L929 cells). (c) Comparison of anti-TNF HuAb and Adalimumab (Wehi 164 Cl. 13 cells). Mean ± SD and nonlinear regression of dose-dependent inhibition of TNF mediated cytotoxicity are shown.

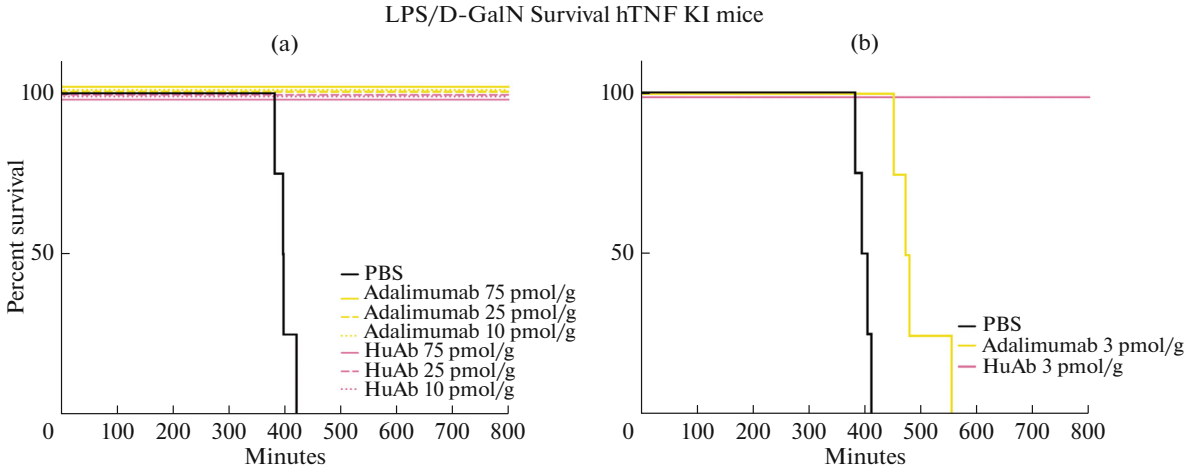

Fig. 6. In vivo efficiency of anti-TNF HuAb compared to Adalimumab. hTNF KI mice ((a) 5 per TNF-inhibitor group, 4 in PBS group; (b) 4 per group) were injected either with diminishing doses of Adalimumab or anti-TNF HuAb or buffer. 30 min later mice were injected with an otherwise lethal dose of LPS/D-Gal. Survival curves for Adalimumab 3 pmol/g and HuAb 3 pmol/g were compared using log-rank test (** $P < 0.01$).

that could be developed as biosimilars to Infliximab and Adalimumab. Although TNF inhibition is highly successful in a fraction of patients, a large proportion of such patients gradually develop immune responses to the immunologically foreign recombinant antibody. Such patient-derived secondary antibodies may block the active site of TNF-inhibitors rendering therapeutics ineffective. Therefore, switching the patient to another TNF-inhibitor may restore therapeutic effect. Accordingly, there is still an unanswered demand for the development of novel TNF inhibitors with unique amino acid sequences.

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