

## Three-Domain Antibodies against the Tumor Necrosis Factor: Investigation of Their Biological Activity In Vitro

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**Abstract**—The aim of this work was to evaluate novel three-domain antibodies consisting of two domains specific for human tumor necrosis factor (hTNF), and of the third domain responsible for binding to myeloid cells. The additional hTNF-binding domain should serve to increase the biological activity of new antibodies. Capacity of these proteins to bind hTNF on the macrophage surface and to neutralize its biological activity in vitro was assessed.

**Keywords:** multispecific antibodies, nanoantibodies, tumor necrosis factor

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### INTRODUCTION

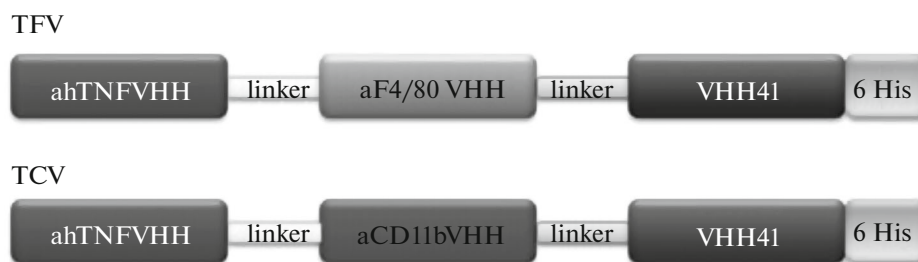
Multispecific antibodies based on the variable domains of the noncanonical antibodies of the Camelidae, the so-called nanoantibodies (VHH), are very promising objects for creating diagnostic and therapeutic agents for treatment of various diseases. The antibody specificity towards two or more types of molecules allows exact localization of a therapeutic target and an increase in efficacy of killing resistant and fast-mutating viruses, for example, the influenza virus [1]. In addition, the directed binding of a target molecule (for example, a tumor-specific marker) to an effector cell can enhance the efficacy of the cancer therapy due to an activation of mechanisms of cellular lysis [2].

The structure of the nanoantibodies opens up new possibilities for their formatting by a fusion of either identical or different monomeric blocks in order to increase avidity or the concomitant blocking of several nonoverlapping epitopes [3]. Therefore, the efficacy of the multivalent complexes is increased in comparison with the monovalent nanoantibodies [4, 5]. For example, bispecific nanoantibodies which simultaneously bind two inflammatory cytokines (for example, TNF and IL-17 [6] or IL-1 and IL-17A [7]) could be promising for a therapy of autoimmune diseases because their therapeutic effect is enhanced in comparison with that of antibodies against one cytokine.

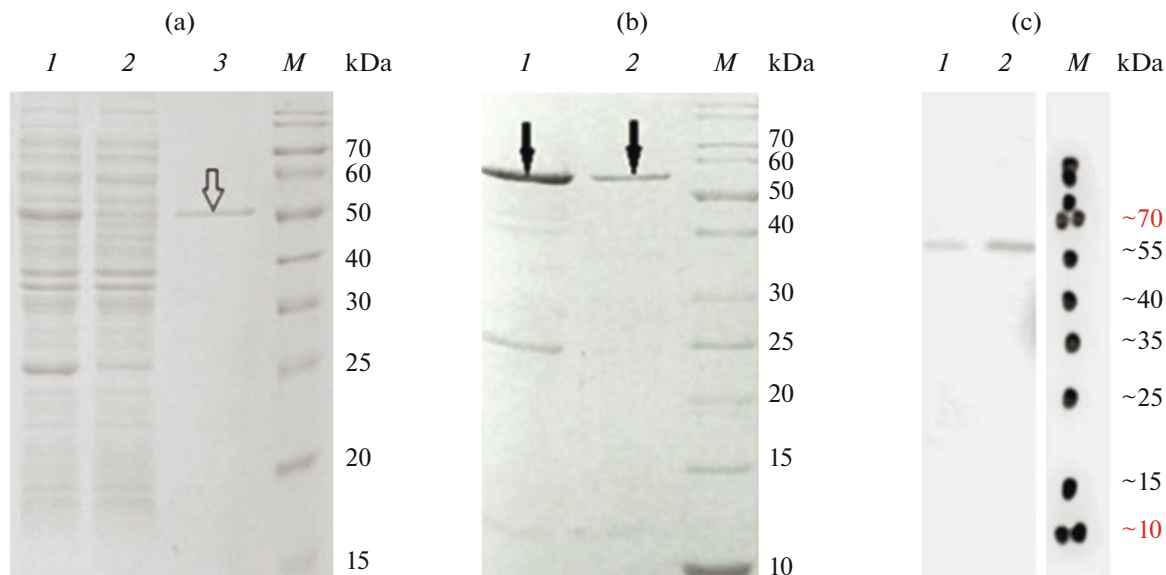
Various cellular receptors which participate in the inflammation [8] can be a target for the second speci-

ficity of the multispecific constructs along with cytokines, for example, molecules on the cellular surface which provide a maximum contribution in an amplification of the inflammatory response in the course of a development of one or other disease. The myeloid cells have been previously shown to be the main source of the tumor necrosis factor (TNF, inflammatory cytokine) in the model of autoimmune arthritis. At the same time, inhibition of the T-cellular TNF results in an aggravation of the disease. Pleiotropic properties of this cytokine provide both protective and “pathogenic” action on functions of an organism and serve the basis of a concept of the need for selective neutralization of TNF [9–11]. Relying on this, the bispecific antibodies which can selectively neutralize TNF from definite sources and have no influence on the protective functions of this cytokine are produced in order to increase the efficacy of the anti-TNF therapy. The bispecific agents have been created on the basis of the nanoantibodies. These agents bind and block hTNF on a surface of the myeloid cells through an interaction with the F4/80 molecule and demonstrate the high efficacy on the model of acute septic shock on the macrophage culture in vitro and on the TNF-gene-humanized mice in vivo [12]. However, a transfer of this model to humans is complicated by the fact that the human F4/80 analog (EMR1) is expressed not only by monocytes/macrophages but, in a large degree, by granulocytes [13] as well. Thus, a search for additional target molecules on the myeloid cells is an important problem in the design of new bispecific antibodies. The CD11b [14] molecule whose Mac-1

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**Fig. 1.** The scheme of the domain composition of bispecific proteins ahTNFVHH–aF4/80VHH–Vhh41 (TFV) and ahTNFVHH–aCD11bVHH–Vhh41 (TCV).



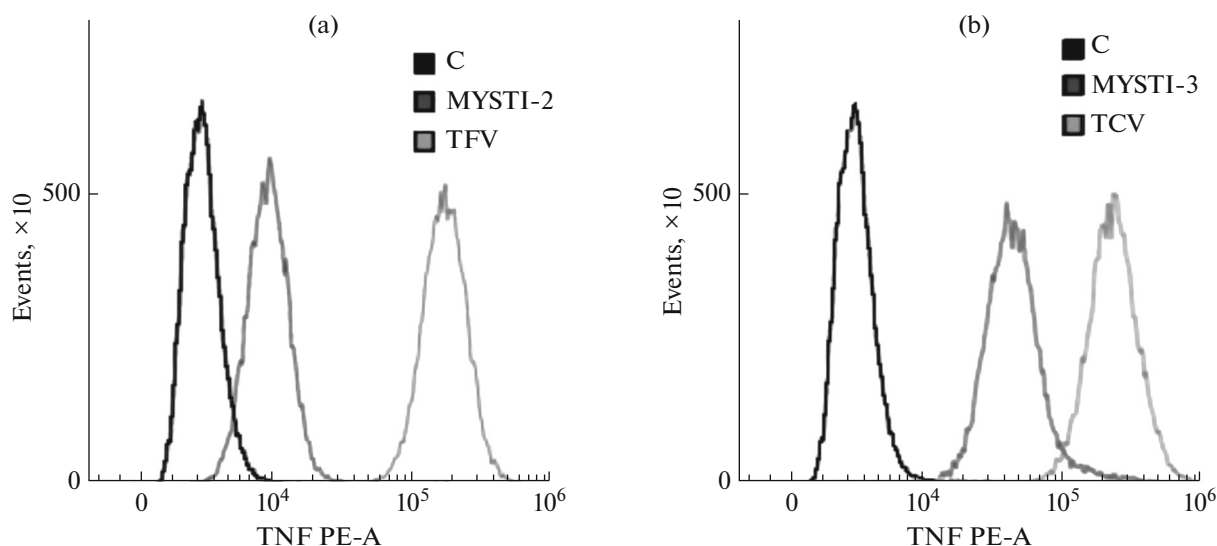
**Fig. 2.** Examples of a typical electrophoregram (SDS-PAGE) and Western blotting of three-domain antibodies: *M*—molecular weight marker (Page Ruler Unstained Protein Ladder, cat. No. 26614, Thermo Fisher Scientific). (a) Samples from different stages of protein purification by the example of TFV by metal chelate affinity chromatography: (1) cell lysate, (2) fraction of proteins not bound during chromatography, (3) TFV eluted protein. (b) Samples of purified proteins TCV (1) and TFV (2). (c) Western blotting of TFV protein samples (1) and TCV (2).

analog is characteristic of the human monocyte/macrophage population and is not produced by eosinophils is chosen as one of such target molecules. Unfortunately, the previously prepared anti-TNF-antibodies which could bind to the CD11b molecule exhibited lower efficacy of the TNF neutralization in comparison with the anti-F4/80 VHH-containing proteins [15]. Therefore, we considered the possibility of introducing the additional TNF-recognizing module (Vhh41) that was bound to TNF with a high affinity [16] into the domain composition of the bispecific antibodies in order to increase their efficacy. Thus, we produced and investigated the three-domain antibodies which were specific to TNF due to the presence of the two different anti-TNF VHH-domains (ahTNF VHH [4] and Vhh41 [16]) and to the surface F4/80 and CD11b markers of the myeloid cells owing to the presence of the aF4/80 VHH or aCD11bVHH domains, respectively.

## RESULTS AND DISCUSSION

Genetic constructions were made for production of the TFV and TCV three-domain antibodies in a bacterial system. The antibody structures were schematically illustrated in Fig. 1. Design of the genetic structures and a preparation of the proteins were described in detail in the Experimental. Homogeneity of the obtained proteins was analyzed by denaturing electrophoresis in PAAG (Figs. 2a and 2b) and western blotting (Fig. 2c).

The binding efficacy of the three-domain antibodies to the F4/80 and CD11b molecules on the macrophage surface was evaluated by flow cytometry. Macrophages for the experiment were obtained from the bone marrow of the TNF-gene-humanized mice in which hTNF was produced instead of the mouse TNF. We analyzed the level of the fluorescent signal from the macrophages that were incubated with the three-



**Fig. 3.** Efficiency of binding of bispecific TNF-blocking proteins to macrophages of mouse TNF (huTNFKI) humanized mice. The level of the fluorescence signal is proportional to the number of analyzed proteins bound to the cells. Comparative fluorescence analysis of cells incubated with: TFV or MYSTI-2 (a), TCV or MYSTI-3 (b). C is the level of autofluorescence signal from cells treated with labeled PE antibodies against human TNF.

domain antibodies or the analogous two-domain antibodies, then, with the recombinant hTNF, and, finally, with detecting fluorescently labeled antibodies to hTNF. The intensity of the fluorescent signal was proportional to the number of macrophages which were bound to the protein blockers. Thus, the signal level indirectly characterized the binding efficacy of the proteins to TNF and the surface proteins on the macrophages. The introduction of the additional module was shown to increase the efficacy of the interaction of the TFV and TCV antibodies with the macrophages in comparison with MYSTI-2 and MYSTI-3 by ten and six times, respectively (Fig. 3).

The ability of the three-domain antibodies to hold the macrophage-produced TNF on the surface of the mouse macrophages was determined according to a quantity of the free TNF in the cellular culture medium by the enzyme immunoassay. The efficacy of the cytokine confinement by the cell-associated bispecific antibodies in this experiment was inversely proportional to the TNF level in the supernatant (Fig. 4). In the control samples (the culture medium from the macrophages without the addition of the bispecific antibodies), the TNF concentration was dramatically increased in the medium after the incubation with lipopolysaccharide (Fig. 4). The lower TNF level was observed for all the examined proteins in comparison with the control, and the TNF quantity in the medium increased with a decrease in the concentration of the bispecific proteins (Fig. 4b). In addition, the three-domain TNF blockers could hold this cytokine on the macrophage surface with the efficacy which was comparable with that of the MYSTI-2 two-domain protein and higher than that of the MYSTI-3 protein.

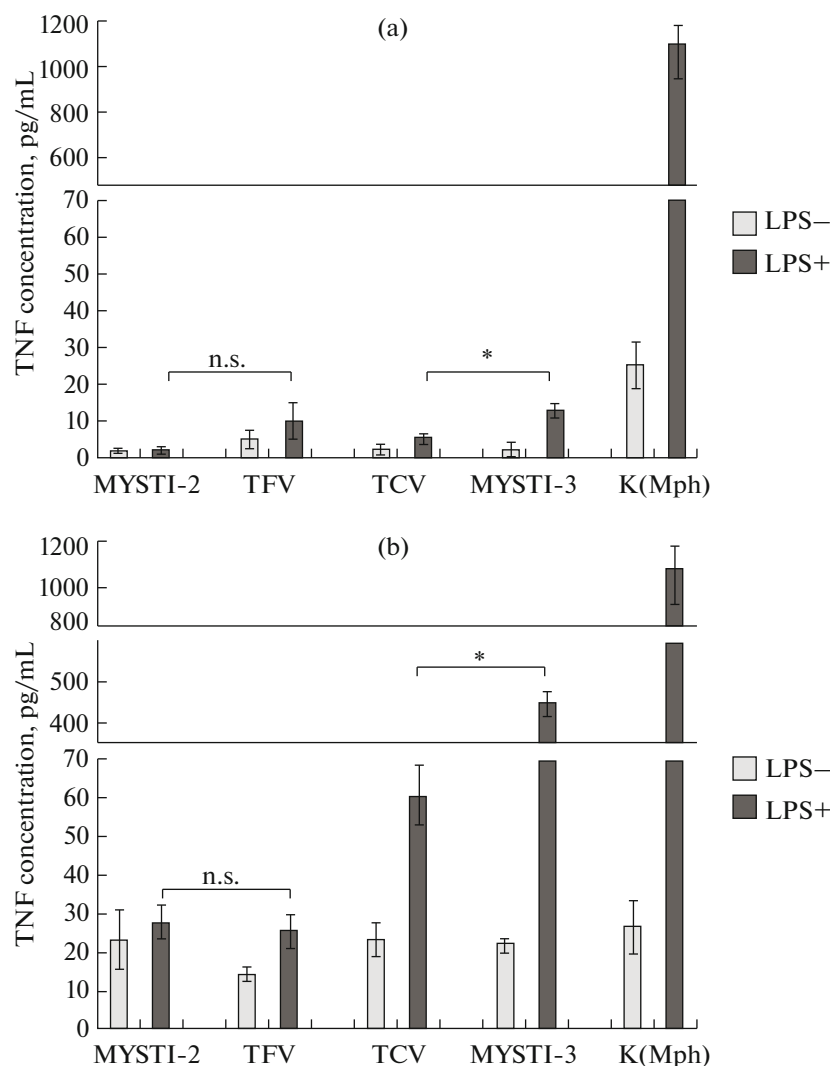
The biological activity of the proteins *in vitro* was studied by the cytotoxic (MTT) test on the cells of the WEHI-164 line (Fig. 5). The most informative characteristics of the biological activity in this test were the  $IC_{50}$  values which proved to be 3.451 and 0.536 nM for the pair of the MYSTI-3 and TCV proteins and 4.5 and 21.9 nM for the pair of the MYSTI-2 and TFV proteins, respectively. Thus, the efficacy of the three-domain protein with the anti-CD11b VHH-domain appeared to be higher than that of its two-domain analog.

Therefore, the introduction of the additional TNF-recognizing module (Vhh41) into the domain composition of the bispecific proteins significantly increased the efficacy of TNF holding on the macrophage surface of the humanized mice. Moreover, the TNF-neutralizing potential of the multispecific construct of the anti-CD11b VHH-containing antibody enhanced in comparison with its two-domain MYSTI-3 analog in the *in vitro* tests.

In general, the results of this study demonstrated that the idea of a construction of bispecific antibodies with an increased number of the TNF-binding sites was promising. This feature of the three-domain antibodies could further be used for the creation of prototypes of drugs for the therapy of inflammatory diseases associated with a hyperexpression of this cytokine.

## EXPERIMENTAL

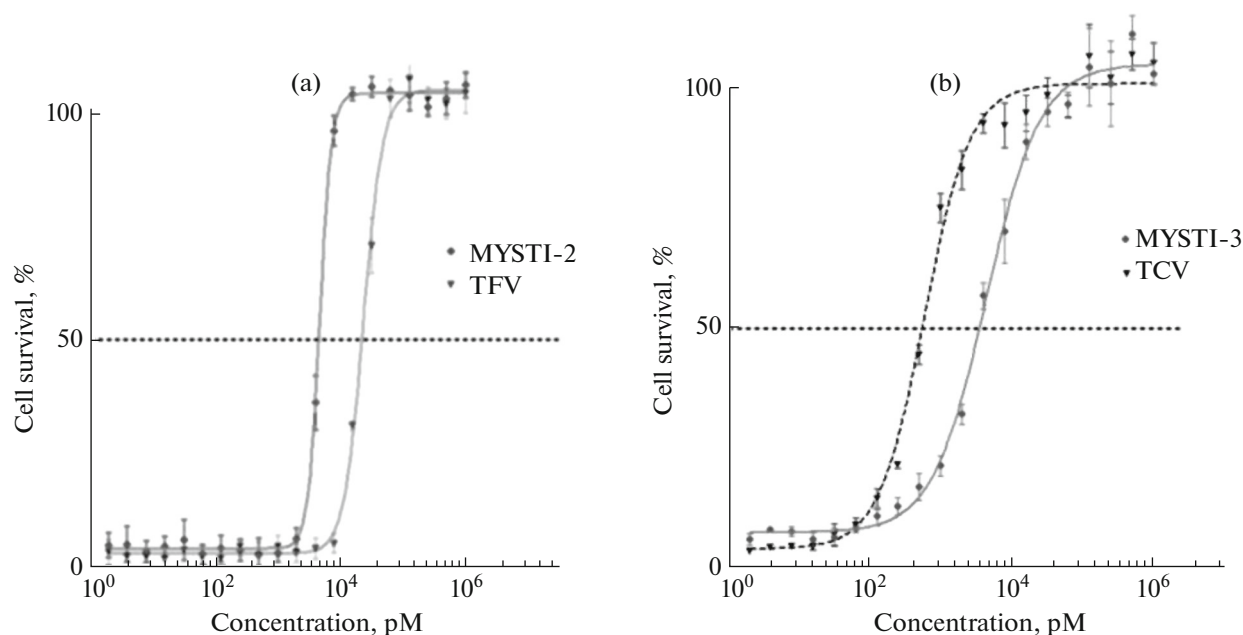
**Materials.** The DH5 $\alpha$  *E. coli* strain with the F $^{-}$  $\phi$ 80lacZ $\Delta$ M15  $\Delta$ (lacZYA-argF)U169 recA1 endA1 hsdR17( $r_K^{-}$ ,  $m_K^{+}$ ) phoA supE44  $\lambda^{-}$  thi-1 gyrA96 relA1 genotype, the Rosetta2(DE3) $\Delta$ SlyD/X *E. coli* strain



**Fig. 4.** Comparison of the amount of free TNF in the culture medium of macrophages incubated with 1 (a) and 0.25  $\mu\text{M}$  (b) of bispecific proteins and lipopolysaccharide. The chart shows the mean values with standard deviations. \*—statistically significant differences ( $p < 0.05$ ), n.s.—statistically insignificant differences.

with the  $F^- \text{ompT hsdS}_B(\text{r}_B^- \text{m}_B^-) \text{gal dcm } \Delta\text{SlyD}/X$  (DE3) pRARE2 (Cam<sup>R</sup>) genotype, the pET28b+ plasmid vector (Novagen), bacto-tryptone (Amresco, United States), sodium chloride (Helicon, Russia), nickel chloride (Helicon, Russia), yeast extract (Amresco, United States), bacto-agar (Difco, United States), agarose (Helicon, Russia),  $\beta$ -mercaptoethanol (Helicon, Russia), Triton X-100 (Panreac Applichem, Germany), acrylamide (Helicon, Russia), methylenebisacrylamide (Helicon, Russia), isopropyl- $\beta$ -D-1-thiogalactopyranoside (Helicon, Russia), EDTA (Serva, Germany), SDS (Panreac Applichem, Germany), imidazole (Panreac Applichem, Germany), dimethyl sulfoxide (Panreac Applichem, Germany), ammonium persulfate (Panreac Applichem, Germany), TEMED (Panreac Applichem, Germany), glycerin (PanReac, Germany), kanamycin

(PAO Biokhimik, Russia), chloramphenicol (Sigma-Aldrich, United States), Ni-NTA Superflow resin for the metal-affinity chromatography (GE Healthcare, Great Britain), Tris (Amresco, United States), HEPES (Amresco, United States), L-arginine (Diaem, Russia), guanidine hydrochloride (Amresco, United States), glutathione oxidized (Amresco, United States), glutathione reduced (Amresco, United States), bovine serum albumin (Panreac Applichem, Germany), Nonfat dried milk powder (Panreac Applichem, Germany), kits for the DNA molecular weight standards (Thermo Scientific, United States), kits for the protein molecular weight standards (Thermo Scientific, United States), the Gibson assembly cloning kit (NEB), BigDye<sup>TM</sup> Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific, United States), Human TNF $\alpha$  ELISA Ready-SET-Go! (MACS, Miltenyi Biotec, Germany), the anti-



**Fig. 5.** Graphs of the dependence of the survival of WEHI-164 cells on the concentration of bispecific antibodies that neutralize the action of exogenous TNF during the cytotoxic (MTT) test. Comparative analysis of TNF-neutralizing activity of TFV and MYSTI-2 (a) proteins, as well as TCV and MYSTI-3 (b) proteins.

4His-HRP antibodies, the West Dura Extended Duration Substrate kits (SuperSignal, United States), ECL Western Blotting Substrate kits (Promega, United States), DPBS (1×) (Gibco), RPMI Medium 1640 (1×) (Gibco), DMEM (1×) (Gibco), HS (horse serum, Gibco), L-glutamine (PanEco, Russia), and the mixture of penicillin and streptomycin (PanEco, Russia) were used in this study.

**The TCV and TFV genetic constructions** which encoded the three-domain TNF blockers were designed with the use of the DNA Star Lasergene software (DNA Star Madison, WI, United States) (Fig. 1). The DNA constructions of the target proteins were prepared by the standard methods of molecular biology. The expression plasmids were produced based on the pET28b vector (Novagene). Sequences of the oligonucleotide primers (Table 1) were synthesized by the OOO DNA-Sintez (Moscow). The DNA sequences of separate cellular clones were analyzed by electrophoresis in 1.5% agarose gel with the subsequent sequencing of the plasmid DNA (ABI Prism 310 Genetic Analyzer). The genetic constructions found were used for a transformation of cells of the Rosetta2-(DE3) $\Delta$ SlyD/X *E. coli* expression strain [17].

**Preparation of the proteins.** Colonies of the Rosetta2(DE3) $\Delta$ SlyD/X *E. coli* were cultured in the LB liquid medium with the selective antibiotics for one night at 37°C and 150–200 rpm, transferred into the fresh LB medium in a ratio of 1 : 10, and cultured for 1.5 h at 37°C and 150 rpm to 0.6–0.7 OU optical density at 600 nm. The expression was induced by isopropyl- $\beta$ -D-1-thiogalactopyranoside (0.5 mM) for 19–20 h at

20°C and 150 rpm. The culture was precipitated by centrifugation at 9000 rpm for 15 min, and the proteins were isolated. The precipitate was resuspended in the buffer containing 25 mM HEPES (pH 7.0), 500 mM sodium chloride, 6 M guanidine hydrochloride, 10% glycerin, 1% Triton X-100, 5 mM imidazole, and 1 mM 2-mercaptoethanol. The suspension was disintegrated by a QSonica Q55 (QSonica sonicators, United States). The lysate was centrifuged for 20–30 min at 9000 rpm and purified by affinity chromatography on a Ni-NTA Superflow sorbent (GEHealthcare, Great Britain). The proteins that were not bond to the chromatographic matrix were removed with the 15-mM solution of imidazole. The proteins were refolded on the column that was eluted with a stepwise gradient of guanidine hydrochloride from 6 M to 0 M using buffer solutions containing 25 mM HEPES (pH 7.0), 500 mM sodium chloride, 10% glycerin, 0.4 M *L*-arginine, 1 mM reduced glutathione and 0.1 mM oxidized glutathione. The chaotropic agent was completely removed, and the proteins were eluted with the following buffer solution: 25 mM HEPES (pH 7.0), 500 mM sodium chloride, 10% glycerin, 0.4 M *L*-arginine, 500 mM imidazole, and 1 mM 2-mercaptoethanol. The proteins after a dialysis were concentrated on an Amicon Ultra 15 concentrator (Merck Millipore Ltd.) with c MWCO 5000 Da. The proteins were analyzed by standard electrophoresis in 12% polyacrylamide gel with subsequent staining with the Coomassie R250 solution [16] and by Western blotting using anti-4His-HRP antibodies (MACS, Miltenyi Biotec, Germany). The results were visual-

**Table 1.** The oligonucleotide primers used in this work

Primer	Sequence
hTNF GC_F	TTAACTTTAAGAAGGAGATATAATGTCCCAAGTTCAGCTTCAAG
hTNF GC_R	CGCCTCCAGAGCCTCCACCGCCAGAGCCACCTCCTCCCGCGCCGGCAGAAGAAACAGTCAC
VHH41 GC_F	CCGCAACCACAACCAAAACCACAACCAAAACCTGAACCAGAAAGCGGATCCATGGCTCAGG-TGCAGCTGGTG
VHH41 GC_R	CGATCCTCGGCCGGAAGCGTAGTCCGGAACGTCGTACGGGTAGCGGCCGCTGGAGAC
pET28 GC_F	TCCGGACTACGCTTCCGGCCGAGGATCGCTCGAGCACCACCACCACCACCTGA
pET28 GC_R	CTTGAAGCTGAACTTGGGACATTATATCTCCTTCTTAAAGTTAA
F4/80 GC_F	GGCTCTGGCGGTGGAGGCTCTGGAGGCGGTGGCTCCATGGCCCAGGTGCAGCTGCAG
F4/80 GC_R	GTTGTGGTTTTGGTTGTGGTTGCGGTTGTGGTTGTGGTTTTGGTTGTGGTTGGGGTATC-TTGGGTTCTGAGGAGACGGTGACCTGGGTCC
CD11b GC_F	TCTGGCGGTGGAGGCTCTGGAGGCGGTGGCTCCATGAGCCAGGTGCAGCTGCAGGAGTC
CD11b GC_R	GTTGTGGTTTTGGTTGTGGTTGCGGTTGTGGTTGTGGTTTTGGTTGTGGTTGGGGTATC-TTGGGTTCCGCCACCGGTTTCCGGCAGTCCTC

ized using West Dura Extended Duration Substrate (SuperSignal, United States).

**Evaluation of the binding of the antibodies to the ligands by flow cytometry.** The macrophages were obtained from the bone marrow (BMDM) of mice that were humanized by the TNF gene [18, 19]. The macrophages were cultured in Petri dishes for 10 days in the DMEM medium containing 20% horse serum (v/v), 30% conditioned medium from the L929 cells (v/v), *L*-glutamine (292 mg/mL), penicillin (100 Units/mL), and streptomycin (100 mg/mL). The cells were washed and removed from the surface of the dishes with an ice-cold phosphate-buffered saline (PBS). The number of the cells was counted using the Goryaev chamber. The dead cells were distinguished from living cells by staining with trypan blue. The Fcγ-receptors were blocked by the TruStain FcX™ (anti-mouse CD16/32) antibodies (cat. No. 101319, Biolegend, United States). The cells were sequentially incubated with the obtained antibodies (4 μM) for 30 min at 4°C, the human recombinant TNF (100 μg/mL) for 30 min at 4°C, and with the phycoerythrin(PE)-labeled antibodies against human TNF-α (Biolegend, United States) for 30 min at 4°C. The samples were analyzed on a Cytotflex S cytometer (Beckman Coulter, United States) using CytExpert 2.0 software (Beckman Coulter, United States).

**Evaluation of the TNF holding by the activated macrophages.** The macrophages (10<sup>5</sup> cells per one well) which were prepared by the method described above were cultured in 96-well plates (Corning® 96-Well Microplates, Corning, United States), incubated for one night at 37°C and 5% CO<sub>2</sub>, washed with the complete DMEM medium, and incubated with the examined proteins (2 μM) or DMEM (as a control) for 30 min at 37°C and 5% CO<sub>2</sub>. The cells were stimulated by lipo-

polysaccharide (LPS, Sigma-Aldrich, L2630) at a dose of 100 ng/mL for 4 h at 37°C and 5% CO<sub>2</sub> for the TNF production. The supernatant was transferred to 96-well plate (Corning Costar, Corning, United States), and the TNF concentration was measured using the HumanTNF-αELISAready-SET-Go® (eBioscience, United States) according to the manufacturer's protocol.

**Cytotoxic MTT test.** The TNF-inhibitory activity of the recombinant proteins obtained was studied using the cytotoxic MTT test on the WEHI-164 cell line sensitive to human TNF [20]. Cells were transferred to 96-well culture plates (Corning® 3788 96-Well Polystyrene Microplates, Corning, United States) at a rate of 2 × 10<sup>3</sup> cells per well. Recombinant hTNF was added at a concentration of 100 u/mL, the studied antibodies in serial dilutions of 1 mM–2 pM. After 24 hours of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, United States) was added at a concentration of 4 μg/mL and incubated for 4 hours, after which the crystals of formazan were dissolved in 10% (w/v) SDS in DMSO with the addition of acetic acid, and the optical density (OD) was measured at 570 nm using a Multiskan EX spectrophotometer (ThermoFisher Scientific, United States). Then, the percentage of living cells was calculated and the values were displayed on nonlinear regression curves using Prism 5 software (GraphPad, United States).

**Statistical processing of results.** Statistical analysis of the results was performed using the Mann–Whitney *U*-test. Differences between groups were considered statistically significant at a significance level of *p* < 0.05.

## ACKNOWLEDGMENTS

Part of the sequencing work was performed on the equipment of the Genom CEC of the IMB RAS ([http://www.eimb.ru/ru1/ckp/ccu\\_genome\\_c.php](http://www.eimb.ru/ru1/ckp/ccu_genome_c.php)).

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## COMPLIANCE WITH ETHICAL STANDARDS

In this work, the international principles of care and use of animals were observed.

*Conflict of Interests*

The authors declare no conflict of interest.

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