The cytokine TNF is a well known drug target for several inflammatory diseases such as Crohn disease. Despite the great success of TNF blockers, therapy could be improved because of high costs and side effects. Selective inhibition of TNF receptor (TNFR) 1 signaling holds the potential to greatly reduce the pro-inflammatory activity of TNF, thereby preserving the advantageous immunomodulatory signals mediated by TNFR2. We generated a selective human TNFR1 inhibitor based on Nano-body (Nb) technology. Two anti-human TNFR1 Nbs were linked to generate Nb Alb-70-96 named “TNF Receptor-One Silencer” (TROS). TROS competes with TNF for binding to human TNFR1. In HEK293T cells, TROS strongly reduces TNF-induced gene expression, like IL8 and TNF, in a dose-dependent manner; and in ex vivo cultured colon biopsies of CD patients, TROS inhibits inflammation. Finally, in liver chimeric humanized mice, TROS antagonizes inflammation in a model of acute TNF-induced liver inflammation, reflected in reduced human IL8 expression in liver and reduced IL6 levels in serum. These results demonstrate the considerable potential of TROS and justify the evaluation of TROS in relevant disease animal models of both acute and chronic inflammation and eventually in patients.

Tumor necrosis factor (TNF) is a pleiotropic cytokine that exerts beneficial activities in immune regulation and host defense, as well as hazardous pro-inflammatory and cytotoxic activities during inflammation. It exists in both membrane-bound and soluble forms (1). TNF signaling is mediated by its binding to one of two different cell-surface receptors as follows: TNF receptor (TNFR)1 (TNFR1 or p55) or TNF receptor 2 (TNFR2 or p75) (2). Although TNFR1 is ubiquitously expressed on most types of cells, TNFR2 expression is inducible and limited mainly to immune, endothelial, and neuronal cells (1). Both TNF receptors are homotrimeric transmembrane glycoproteins. The extracellular domains of both receptors are conserved and consist of a ligand-binding part composed of a ligand-binding domain and plays many roles in cell survival and proliferation (5). Furthermore, TNFR2 plays a role in immune regulation and in maintaining tissue homeostasis, for example by activating Treg cells (3, 5).

The TNFR ligand is a well known drug target for several inflammatory diseases such as rheumatoid arthritis (RA) and Crohn disease (CD). Anti-TNF antibodies or antibody fragments such as infliximab, adalimumab, certolizumab, and etanercept are used to treat those diseases, and they are among...
Nanobodies That Inhibit TNFR1

Isolation of hTNFR1-specific Nbs—To screen for the presence of human TNFR1-specific Nbs, four consecutive rounds of panning were performed on solid-phase coated hTNFR1 (100 μg/ml, 10 μg/well). The enrichment for antigen-specific phages after each round of panning was assessed by polyclonal phage ELISA. Enrichment was obtained after the 3rd and clearly after the 4th round of panning. We randomly selected 190 colonies after the 3rd round and 142 after the 4th and analyzed them by ELISA for the presence of antigen-specific VHIs in periplasmic extracts. Of 332 colonies, 34 scored positive in this assay (7/190 and 27/142 from 3rd and 4th rounds, respectively). The selected clones were analyzed, and their VHH genes were sequenced to identify the different Nbs.

Subcloning, Expression, and Purification—The vh genes of the selected clones were subcloned from pHEN4 into the pHEN6c expression vector, in fusion with a C-terminal His6 tag, using PstI and BstEI (Promega) (23). To generate TROS, the pHEN6c vector was transformed into WK6 Escherichia coli cells, and the Nb expression was induced as described previously (Fig. 1B) (23). The expressed Nbs were extracted from the periplasm by osmotic shock (24) and purified using a nickel-Sepharose 6 FF column (GE Healthcare), equilibrated with 20 mM NaH2PO4, pH 7.5, 500 mM NaCl, 20 mM imidazole, and 1 mM PMSF. After loading, the column was washed with 20 column volumes of the same buffer in the presence of 0.1% empigen as detergent. Before elution, the column was equilibrated with 5 column volumes of equilibration buffer without detergent. The Nbs were first eluted with 20 mM NaH2PO4, pH 7.5, 20 mM NaCl, 50 mM imidazole, and 1 mM PMSF and then with 400 mM imidazole in the same buffer. The eluate was diluted 20 times with 25 mM sodium acetate, pH 5.5, and loaded on a Source 15S column (GE Healthcare) to remove LPS and other contaminants. After equilibration, the Nbs were eluted by a linear gradient over 20 column volumes of NaCl from 0 to 1000 mM in 25 mM sodium acetate, pH 5.5. Finally, the recombinant protein was injected on a Superdex 75 gel filtration column with PBS as running solution. The obtained fractions were analyzed by Coomassiestained SDS-polyacrylamide gels and anti-His Western blots. Protein concentration was measured by the Micro-BCA assay (Pierce). LPS levels were determined using an EndoSafe-PTS assay (Charles River) that makes use of LAL reagents in a Food and Drug Administration-licensed disposable test cartridge with a handheld reader for real time endotoxin testing. The LPS concentration was <0.5 EU/ml.

Cloning and Expression of TROS—To generate the trivalent Nb “Alb-70-96” (TROS), an albumin-binding Nb (25) was linked to Nb 70 and Nb 96 by a (Gly4-Ser)3 sequence. First, we generated the bivalent Nb 70–96 construct. The Nb 70 VHH gene was amplified by PCR using a sense bivalent Nb primer (5'-GGCCAGGCGCCCATGGCCAGKTCGAGCTACAGGAGTNGGNGG-3') and an antisense bivalent Nb primer (5'-GGCCAATTCC-TGCAGTCTGCAGCTACTCCGCGAGCATGGAGGTAATCCGACCTGGGT-3'). The amplified Nb 70 gene and the pHEN6c vector containing Nb 96 were digested with PstI and NcoI (Promega). Next, we ligated Nb 70 into the pHEN6c vector containing Nb 96, and the ligation product was transformed into E. coli.
strain WK6. Positive colonies were screened by PCR and sequenced to validate the sequence of the bivalent Nb 70–96. To obtain a TROS-containing construct, this procedure was repeated by ligating Nb Alb VHH into the pHEN6c vector containing Nb 70–96.

To increase the expression yield of TROS, we used the eukaryotic yeast *Pichia pastoris*. The TROS-encoding sequence was cloned into the pAOXZalpha vector, a derivative of the pPICZα vector from Invitrogen. The expression vector contains the *aox1* promoter fused to the α-mating factor pre-pro signal sequence followed by the gene coding for the Nb. The Nb contained a His₆ tag at the C terminus comparable with the *E. coli* construct (Fig. 1C). First, the Nb gene was amplified by PCR using primers pAOXpeliG (5′-TCTCTCGAGAAAAAGT-TATTACTCGGGCCAGCGG-3′) and HISpAOX (5′-CAAGCTTAGATCTATTTGATGGTGGTGTT-3′). The Nb gene was then digested with Xhol and HindIII (Promega), and the pAOXZalpha vector was digested with Xhol/Xmal followed by digestion with HindIII/Xmal (Promega). The Nb gene was then ligated in pAOXZalpha, using a three-point ligation. pAOXZalpha Nb Alb-70-96 vector was transformed in *P. pastoris* and sequenced to validate the sequence of the bivalent Nb 70–96.

The Nb affinity for wild type GS115 ligation. pAOXZalpha Nb Alb-70-96 vector was transformed in *E. coli* using a mixture of *N*-hydroxysuccinimide and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride until a response unit of 581 was obtained at 25 °C. Binding experiments were performed at 25 °C in HBS (10 mM Hepes, pH 7.5, 150 mM NaCl, 3.5 mM EDTA, and 0.005% Tween 20) at a flow rate of 20 μl/min, applying a 2-fold dilution series of Nb ranging from 500 to 1 nM and an hTNFR1 Ab dilution series from 250 to 1 nM as positive control. Between applying different Nb concentrations, the chip was regenerated during 300 s at a flow rate of 30 μl/min with 25 mM NaOH and 500 mM NaCl, without any detectable effect on the binding capacity of hTNFR1. A blank uncoated channel was used as an online reference during all injections. To analyze the results and determine the kinetics of association (kₐ) and dissociation (kₐ), the sensograms were fitted by subtracting the signal of the reference flow cell using BIACore T200 software. Nb 70 and Nb 96 were fitted with a 1:1 binding model, whereas curves of Nb TROS were fitted with a bivalent binding model.

SPR was also used to perform competition assays and to determine whether Nb 70, Nb 96, and TNF bind the same epitope(s). hTNFR1 Ab was used as a positive control. The flow conditions as described above were used. First, a 450-s binding phase with either Nb or the hTNFR1 Ab at saturating concentrations (1 μM) was followed by a second 450-s binding phase with a mixture of Nb or hTNFR1 Ab and competitor (TNF), and vice versa. Curves were analyzed using evaluation software (BIACore) and interpreted visually.

**Docking Models**—To predict Nb-hTNFR1 binding, homology modeling and docking were performed. A homology model of trimeric hTNFR1 was built with Modeler (27) using monomeric hTNFR1 (PDB code 1TNR) and trimeric hTNFR2 (PDB code 3ALQ) as templates. Homology models of all Nbs were also generated by Modeler using multiple templates from PDB, namely 4FZE, 4JVP, 3P0G, and 2KH2 (28). All models were validated by RAMPAGE (29), and the best models were used for docking by ClusPro (30) to predict binding of Nb 70 and Nb 96 to hTNFR1. Homology models and docking results were analyzed, and figures were rendered using PyMOL.
Serum Half-life—To determine the clearance of TROS after intravenous injection, 8-week-old female C57BL/6J mice (January) were intravenously injected with 100 μg of TROS in a total volume of 300 μl of PBS. Blood samples of alternating mice were taken retro-orbitally after 1, 3, and 8 h, and then daily until day 8. Blood was stored overnight at 4 °C, and supernatant was collected from clotted blood and centrifuged at 14,000 × g for 15 min at 4 °C. Nb serum concentrations were determined by hTNFR1 ELISA as described above.

To determine the clearance of TROS after intraperitoneal injection, a mixture of ten-week-old male and female C57BL/6J mice (own breeding) were intraperitoneally injected with 200 μg of TROS in a total volume of 300 μl of PBS. Blood samples were taken retro-orbitally after 1, 3, 7, and 10 h and then daily until day 6. Blood was analyzed as described above.

Inhibition of NF-κB-dependent Genes and Cytokines Induced by TNF—HEK293T cells were seeded at 500,000 cells per well in a 6-well plate in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. The next day, cells were preincubated with 1 μM Nb (TROS or Nb Alb-Ctrl-Ctrl), 1 μM etanercept (ETA), or 0.2 μM hTNFR1 Ab as a positive control and dissolved in 100 μl of medium, for 30 min at 37 °C. Subsequently, 1000 IU/ml hTNF was added. To determine the therapeutic effect of TROS, the reversed situation was also performed. Here, cells were first incubated with hTNF for 30 min at 37 °C followed by addition of the NbS or Abs. Six hours after TNF stimulation, RNA was isolated using TRizol and the InviTrap Spin Universal RNA mini kit (Isogen Life Science). qPCR was performed as described above. qPCR was performed using primers for A20, ICAM (ICAM1), VCAm (VCAM1), and IL8, and expression levels were normalized to housekeeping genes UBC and 28S, selected with GeNorm. All primers were human-specific and do not cross-react with the murine equivalent. Serum was prepared from blood as described above and a human-specific IL6 ELISA (IL6 ready-set-go ELISA, eBioScience) was performed to measure human IL6 levels in serum.

Ethics Approval for Animal and Human Studies—All animal experiments were approved by the ethics committee of the Faculty of Science of Ghent University or the Faculty of Medicine and Health Sciences of Ghent University. Mice were housed in a specific pathogen-free animal facility with 12-h light and dark cycles and free access to food and water. The study on human colonic biopsies was approved by the ethics committee of Ghent University Hospital (permit number EC UZG 2004/242), and each participant provided a signed informed consent form.

Statistical Analysis—Data are presented as means ± S.E. Data were analyzed with an unpaired t test, unless mentioned differently. Significance levels were calculated for differences between TROS, vehicle, and Nb Alb-Ctrl-Ctrl, as indicated (*, 0.01 ≤ p < 0.05; **, 0.001 ≤ p < 0.01; ***, 0.001 ≤ p < 0.0001; ****, p < 0.0001).

RESULTS

Generation of Anti-hTNFR1 Nbs—An alpaca (V. pacos) was immunized with recombinant human soluble TNFR1 protein, according to standard procedures. Next to conventional antibodies (IgG1 subclass), alpacas produce antibodies with only heavy chains (IgG2 and IgG3 subclasses) in response to the recombinant protein (18). The immunized alpaca generated an immune response in all IgG subclasses. The IgG2 and IgG3 responses were weaker than the IgG1 response. Among the heavy-chain-only antibodies, the IgG3 response was the strongest. Subsequently, a PHEN4 phagemid library was generated from blood B-lymphocytes isolated from the immunized alpaca (35) and transformed in E. coli TG1 cells. A library of about 10⁸ independent transformants was obtained, and about 74% of them harbored the vector with the right insert size. Next, four consecutive rounds of panning with the phage-displayed Nbs on solid-phase coated hTNFR1 were performed. Enrichment was obtained after the 3rd and clearly after the 4th round, after which a total of 332 individual colonies (190 and 142 after 3rd
and 4th rounds of panning, respectively) were randomly selected and analyzed by hTNFR1 ELISA for the presence of antigen-specific VHHs in their periplasmic extracts. Sequencing of VHH genes from 34 positive colonies resulted in eight different specific VHHs in their periplasmic extracts. Sequencing of VHH genes from these colonies revealed that Nb 19, 20, 96, and 9 are from clonally related B-cells resulting from somatic hypermutations. Nb 23, 70, and 22 belong to unrelated B-cell clones. Gaps (dashes) are introduced to align sequences. As expected, the Nb Ctrl did not bind hTNFR1.

Analysis of the hTNFR1 Binding and Inhibition of the Different Anti-TNFR1 Nbs—We determined the binding efficiency of the monovalent anti-hTNFR1 Nbs to hTNFR1 by ELISA, starting at a saturating concentration of 2.7 μM. This allowed us to identify the Nbs with the highest affinity (Fig. 2A). A hTNFR1 monoclonal antibody (Ab) was used as a positive control. cAbBcII10 (Nb Ctrl) is an irrelevant monovalent Nb that served as a negative control (23). Nb 96 and Nb 70 bound with the highest affinity to hTNFR1 (Kd 34.8 and 55.61 nM, respectively), followed by Nb 70. None of the other Nbs showed high binding affinity to hTNFR1 (Kd > 105 and > 6.09 × 105 nM, respectively), in accordance with their lower affinity, as expected, the Nb Ctrl did not bind hTNFR1.

Next, we used HEK-2 blue cells to determine the ability of Nb 96 and Nb 70 to inhibit the TNF/TNFR1 signaling cascade. Here, TNF/TNFR1 signaling activates NF-κB (38), and this results in the transcription and secretion of the reporter protein alkaline phosphatase, which can be detected by purple/blue coloration of the HEK-2 blue detection medium. We used this system in the presence of increasing concentrations of the different Nbs. Fig. 2B shows that Nb 70 is the only Nb that inhibits TNF/TNFR1 signaling within the tested dose range.

We then studied the binding kinetics of Nb 96 and Nb 70 on immobilized hTNFR1 using SPR. SPR enables the analysis of direct biomolecular interactions with several advantages over traditional methods, such as speed, no need for labeling, real time, and use of micro-samples (39). SPR analysis revealed that both Nb 96 and Nb 70 bind with high affinity to immobilized hTNFR1 (Fig. 2, C and D). Their rapid binding to hTNFR1 is reflected in the high association constants (kₐ, 6.09 × 10² and
but because they are mono-valent, they also quickly dissociate from the receptor, reflected by a relatively high \( k_d \), namely 0.02357 and 0.03806 s\(^{-1}\), respectively. Nevertheless, as summarized in Table 1, their overall equilibrium affinities (\( K_D \)) are in the low nanomolar range, 38.71 and 11.59 nM, for Nb 96 and Nb 70 respectively.

3.282 \( \times 10^6 \text{M}^{-1} \text{s}^{-1} \), respectively), but because they are monovalent, they also quickly dissociate from the receptor, reflected by a relatively high \( k_d \), namely 0.02357 and 0.03806 s\(^{-1}\), respectively. Nevertheless, as summarized in Table 1, their overall equilibrium affinities (\( K_D \)) are in the low nanomolar range, 38.71 and 11.59 nM, for Nb 96 and Nb 70 respectively.

**Epitope Mapping of Monomeric Nb-Binding Sites and in Vitro Competition with TNF**—We used SPR to identify the hTNFR1-binding epitopes of Nb 70 and Nb 96. Nb 70 was added to the immobilized hTNFR1 at saturating concentrations followed by Nb 96, and vice versa. This enabled us to study competition between the Nbs for binding to hTNFR1. Fig. 3A

**TABLE 1**

|                | SPR-analysis\(^1\) | K\(_D\) ELISA\(^2\) | SPR-competition assay\(^3\) |
|----------------|--------------------|---------------------|-----------------------------|
|                | \( k_a \) \( M^2 \text{s}^{-1} \) | \( k_d \) \( s^{-1} \) | hTNFR1 | Albumin | Competition? |
| Nb 70          | 6.09 \( \times 10^5 \) | 0.02357 | nM | 38.71 | 7.709 | NA | TNF |
| Nb 96          | 3.282 \( \times 10^6 \) | 0.03806 | 11.59 | 4.198 | NA | None |
|                | \( k_{d1} \) \( s^{-1} \) | \( k_{d2} \) \( s^{-1} \) | \( k_d \) \( M^2 \text{s}^{-1} \) | hTNFR1 | Albumin | Competition? |
| TROS           | 2.844 \( \times 10^5 \) | 199.71 | 0.01344 | 513.7 | 5.66 | 0.1151 | 0.6147 | TNF |

**FIGURE 2.** A–D, binding of hTNFR1 and inhibition of TNF/hTNFR1 signaling by the monovalent nanobodies and surface plasmon resonance sensorgrams of Nb 96 and Nb 70 binding to immobilized hTNFR1. A, to determine the binding affinity, an hTNFR1 ELISA with the monovalent Nbs was performed. A serial 0.2 dilution was applied, starting at 2.7 \( \mu M \). B, using a HEK-2 blue assay, the inhibition capacity of TNF/hTNFR1 signaling by the monovalent Nbs was determined. HEK-2 blue cells were preincubated with a 0.2 Nb dilution series starting at 2.7 \( \mu M \) and stimulated with 1000 IU/ml TNF. Nb 70 was identified as the only inhibiting Nb. C and D, SPR analysis of Nb 96 and Nb 70. The adjusted sensorgrams overlays show binding of Nb 96 or Nb 70 applied in a dilution series from 1.95 to 500 nM to immobilized hTNFR1. Dotted lines show global fitting of the binding data to a 1:1 interaction model. Both Nbs have good association constants (high \( k_a \)), but show quick dissociation (\( k_d \)). Nb Alb-Ctrl-Ctrl, an irrelevant control Nanobody; hTNFR1 Ab, a human TNFR1 antibody, positive control. Black dotted line, Nanobody concentration that binds 50% of hTNFR1 or albumin. The ELISA and HEK-2 blue assay were done in triplicate, and data are represented as mean \( \pm \) S.E. Surface plasmon resonance analyses were done in duplicate.
illustrates that Nb 70 and Nb 96 can bind simultaneously to hTNFR1, as the signal obtained by binding of a mixture of the two is the sum of the signals obtained from each Nb individually. This means that they recognize and bind different hTNFR1 epitopes. Next, we determined whether the Nbs compete with TNF binding. First, either Nb 70 or Nb 96 was added, followed by TNF, and vice versa. The sensorgrams in Fig. 3B indicate that Nb 70 and TNF compete for binding to hTNFR1, but Nb 96 and TNF do not compete. This suggests that Nb 70 and TNF share the same hTNFR1 epitope, although Nb 96 does not (Fig. 3C). This shows that the binding sites of Nb 70 and TNF for hTNFR1 overlap or are identical and explains the inhibitory, competitive activity of Nb 70, in contrast to Nb 96.

Docking Models of Nb 70 and Nb 96 to Trimerized hTNFR1—
To gain insight into the binding of Nb 70 and Nb 96 to trimeric hTNFR1, homology and docking modeling were conducted. Based on known template structures of monomeric hTNFR1 (PDB code 1TNR) and trimeric hTNFR2 (PDB code 3ALQ), a homology model for trimeric hTNFR1 was designed. Homology models of Nb 70 and Nb 96 were also generated using multiple templates (PDB code 4FZE, 4JVP, 3P0G, and 2KH2). Based on these models, prediction of Nb binding to trimeric hTNFR1, interacting with trimerized TNF, was made. The model of Nb 70 docking to hTNFR1 (Fig. 4A) predicts that Nb 70 binds between two chains of the extracellular domain of trimeric hTNFR1 and interferes with the binding of trimerized TNF to hTNFR1. In contrast, modeling predicts that Nb 96 binds to hTNFR1 near the region that interacts with the cell membrane and so it does not disturb TNF/TNFR1 binding (Fig. 4B). Those predictions are consistent with the SPR competition assays. Additionally, Fig. 4, A and B, shows that the closest contacts between Nb 70 or Nb 96 and trimeric hTNFR1 are through the CDR3 loop of the Nbs.

SPR analysis of competition between Nb 70 and Nb 96 showed that the two Nbs do not compete for hTNFR1 binding because they bind different epitopes. Hence, we generated a prediction model in which both Nb 70 and Nb 96 were docked to trimeric hTNFR1 simultaneously (Fig. 4C). As expected, the two Nbs bind hTNFR1 differently. Moreover, they not only bind different regions of the trimeric hTNFR1, but also interact with different TNFR1 chains as follows: Nb 70 binds chains A and B, and Nb 96 binds chains B and C, and no interchange is possible.

Generation of Multivalent Nbs Expressed in P. pastoris—We generated Nb Alb-70-96 (TROS) consisting of Nb 70 (the only inhibitor of TNF/hTNFR1 signaling), Nb 96 (Nb with highest affinity), and an anti-albumin Nb, linked to each other with flexible (G4-S)3 linkers. As shown in Fig. 4D, linking the C-terminal end of an anti-albumin Nb to the N-terminal end of Nb 70 and its C-terminal end to the N-terminal end of Nb 96 might increase the inhibitory activity of the resulting TROS Nb. Based on the docking model, the flexible linker between Nb 70 and Nb 96 goes through the different chains of trimeric hTNFR1, disturbing its interaction with TNF even more, which might lead to a Nb with better inhibition capacities. Also, the docking model predicts that
In Vitro Characterization of Binding and Inhibition Properties of TROS—The avidity of the trivalent TROS is higher than that of the monovalent Nbs, which might lead to increased affinity. Additionally, changing the expression system from E. coli to P. pastoris might alter the affinity due to changes in protein folding, which thus can lead to altered protein characteristics. Therefore, we characterized TROS affinity by ELISA and SPR. In parallel, we studied the albumin binding properties of TROS and Nb Alb-Ctrl-Ctrl to confirm the functionality of the anti-albumin Nb module after combining it with two other Nbs. As depicted in Fig. 5A, TROS binds to hTNFR1 with a nanomolar affinity \((K_d 0.1151 \text{ nM})\), which is strongly increased in the multivalent format compared with the monovalent Nbs \(((G_4-S)_3 \text{Nb 70 7.709 nM and } K_d \text{Nb 96 4.158 nM})\) (Table 1). Fig. 5B confirms the albumin binding capacity of the anti-albumin Nb in TROS and Nb Alb-Ctrl-Ctrl \((K_d \text{TROS 0.6147 nM and } K_d \text{Nb Alb-Ctrl-Ctrl 0.1015 nM versus } K_d \text{Nb Alb 0.1225 nM})\).

We conducted a new SPR experiment to confirm the increased affinity of TROS relative to the monomeric Nbs. As shown in Fig. 6A, TROS shows rapid kinetics of association \((k_a)\) to immobilized hTNFR1, which is consistent with the results obtained with monovalent Nb 70 and Nb 96. Additionally, although Nb 70 and Nb 96 have high dissociation rates \((k_d)\), the trivalent Nb had a greatly improved \(k_d\) value, which enhances the overall equilibrium constant \((K_d)\). The \(K_d\) of TROS is two times lower than that of Nb 96 and more than six times lower compared with Nb 70 (Table 1). SPR was conducted with hTNFR1 Ab as a positive control, which has higher avidity and therefore a very low \(K_d\) (data not shown).

Not only TNF but also LTα binds TNFR1 and signal through it. Therefore, we tested the effect of TROS on LTα/TNFR1 signaling using the HEK-2 blue assay. Interestingly, preincubation with TROS or Nb 70 inhibited the LTα/TNFR1 signaling, in contrast to the hTNFR1 Ab (Fig. 6D). Finally, in agreement with the known high specificity of Nbs, we excluded by ELISA any cross-reactivity with mouse TNFR1 (mTNFR1) and human TNFR2 (hTNFR2). As shown in Fig. 5, C and D, TROS does not cross-react with mTNFR1 or hTNFR2, in contrast to the hTNFR1 Ab, which also binds to hTNFR2 and mTNFR1.

Competition between TROS and TNF—Assays of competition between TNF and the monovalent Nbs using SPR revealed that Nb 70 competes with TNF for binding to hTNFR1. Because TROS is three times larger than the monovalent Nbs, steric hindrance might increase or decrease this competition. So we examined by SPR analysis whether this competition was maintained after the generation of TROS. TROS and TNF were added consecutively and in reverse order at saturating concentrations \((1 \mu \text{M})\), and competition between the two molecules for binding to hTNFR1 was assessed. The sensorgram in Fig. 6B confirms that TROS competes with TNF for binding to hTNFR1. A hTNFR1 Ab used as a positive control also competes with TNF for hTNFR1 binding (data not shown).
Analysis of the Serum Half-life of TROS—Serum half-life of TROS was determined after a single intravenous (i.v.) injection of 100 μg and a single intraperitoneal (i.p.) injection of 200 μg of TROS in wild type C57BL/6J mice. Blood was taken at various time points for 8 consecutive days, and serum concentrations of TROS were determined using hTNFR1 ELISA. After i.v. injection, TROS shows a biphasic elimination, starting with a distribution and elimination phase that quickly decreases serum TROS concentration, followed by slower elimination from the circulation. Because of the presence of the anti-albumin binding Nb, the serum half-life of TROS is about 24 h, which is significantly longer than the serum half-life of monovalent Nbs (about 1.5 h) (Fig. 7A) (41). After intraperitoneal injection, TROS is absorbed through the peritoneum into the bloodstream (absorption phase), and TROS serum levels reach a maximal concentration (C_max) of 92 μg/ml after 10 h (Fig. 7B). Once C_max is reached, TROS shows similar elimination kinetics compared with i.v.-injected TROS, starting with a quick decrease in serum concentrations followed by a more slower elimination. The serum half-life of TROS after intraperitoneal injection is ∼30 h.

In Vitro Antagonistic Activity of TROS in HEK293T Cells—We further investigated the inhibitory effect of TROS in HEK293T cells by determining the TNF-induced expression of IL8, A20 (TNFAIP3), IκBα, and TNF and IL8 secretion in the presence or absence of 1 μM TROS, and we compared it with the effects of ETA. Incubation with 1000 IU/ml TNF strongly induced gene expression of all tested genes after 6 h and resulted in high IL8 levels in the cell supernatant after 24 h, reaching levels of about 120 pg/ml (data not shown). Preincubation with 1 μM TROS or ETA significantly prevented the up-regulation of all genes tested and resulted in a large reduction of IL8 in the supernatant of HEK293T cells relative to Nb Alb-Ctrl-Ctrl (data not shown). Interestingly, the addition of 1 μM TROS or ETA, 30 min after TNF was applied, could significantly reduce the IL8 and TNF up-regulation as shown in Fig. 8, A and B (other data not shown). In this experimental setup, the efficacy of TROS did not differ significantly with the efficacy of ETA. Preincubation of HEK293T cells with different concentrations of TROS (Figs. 8C and 9D, and other data not shown) showed that this inhibition was dose-dependent with IC50 values of 130.1 nM for inhibition of IL8 gene expression up-regulation and 176.1 nM for inhibition of IL8 secretion. Table 2 summarizes the IC50 values for the other genes tested.

Ex Vivo Inhibition of Inflammation in Biopsies of Inflamed Human Colon—Two biopsies were isolated from the same inflamed region of the colon from patients having acute Crohn disease. The biopsies were cultured ex vivo, one with TROS and the other with Nb Alb-Ctrl-Ctrl followed by mRNA and supernatant isolation. Biopsies from inflamed regions already
showed an inflammatory signature, reflected in high IL8, IL6, and TNF expression and IL8, IL6, and TNF secretion. Incubation with TROS resulted in a substantial decrease in IL8, IL6, and TNF mRNA expression compared with incubation with Nb Alb-Ctrl-Ctrl (Fig. 9, A–C). This was also reflected in decreased secretion of these mediators in the supernatant (Fig. 9, D–F).

In Vivo Inhibition of TNF-induced Liver Inflammation in Mice—Finally, we analyzed the efficacy of TROS in an in vivo mouse model of acute TNF-induced liver toxicity (42). TROS binds and inhibits only human TNFR1 without cross-reacting with mouse TNFR1, so we used transgenic uPA−/−/H11001−/−/SCID mice of which the liver is repopulated with primary human hepatocytes (humanized mice) (32). Because the mice used in this study displayed ~40–60% of liver humanization, we generated qPCR primers that specifically amplify the human genes but not their mouse equivalents. Humanized mice were injected with TNF (6.7 mg/kg) in the presence or absence of TROS pretreatment (200 µg TROS, 30 min before TNF injection), and livers were isolated 2 h later. TNF injection resulted in increased expression of human IL8, ICAM, VCAM, and A20 in the liver and up-regulation of serum IL6 levels (Fig. 10, A and B, and other data not shown). Pretreatment with TROS (13.35 mg/kg, injected intraperitoneally) significantly reduced both liver inflammation (reflected by a decrease in IL8, ICAM, VCAM, and A20 mRNA expression) and systemic inflammation (reflected in a TROS-dependent reduction in TNF-induced human IL6 in serum) (Fig. 10, A and B, and other data not shown).

DISCUSSION

Current TNF inhibitors, including the biologicals IFX, ADA, certolizumab pegol, and ETA, are extensively used to treat diseases such as inflammatory bowel disease, psoriasis, and RA, and they are very useful for patients with active disease failing to
respond to conventional therapy. Unfortunately, these biologicals are very expensive, and some patients suffer from side effects. Additionally, despite their efficacy for RA and inflammatory bowel disease, a major issue in treatment with TNF inhibitors is the lack of (enough) primary response in both diseases (43). Clinical studies on the use of IFX, ADA, and certolizumab pegol for inflammatory bowel disease reported a response rate of about 70% of the patients to initial treatment, but only about 30% of them achieved clinical remission (44). This highlights the urgent need for new therapies with better outcome and response rates. In light of this, specific TNFR1 targeting might be a better approach than complete TNF inhibition (3).

Here, we describe the development of TROS, a TNFR1 antagonist based on Nb technology. Nbs consist of only one single variable domain (VHH), derived from heavy-chain-only anti-

FIGURE 7. A and B, serum half-life of TROS. A, serum half-life of TROS was determined after a single i.v. injection of 100 μg of TROS in wild type C57BL/6J mice (n = 8). Serum concentrations were determined by ELISA. TROS has a C_{max} of −60 μg/ml and has a biphasic elimination. The half-life of TROS (t_{1/2}) is −24 h. B, serum half-life of TROS was determined after a single intraperitoneal injection of 200 μg of TROS in wild type C57BL/6J mice (n = 8). Serum concentrations were determined by ELISA. TROS first shows an absorption phase and after 10 h reaches a C_{max} of −92 μg/ml. Next, TROS is eliminated from the circulation and has a biphasic elimination. The t_{1/2} is −30 h. Data represent mean ± S.E.

FIGURE 8. A–D, inhibition of TNF-induced inflammation in HEK293T cells by TROS. A and B, inhibition of TNF-induced up-regulation of IL8 and TNF by TROS and ETA in HEK293T cells relative to Nb Alb-Ctrl-Ctrl. HEK293T cells were first stimulated with hTNF (1000 IU/ml) and after 30 min incubated with 1 μM TROS or ETA for 6 h. C and D, to determine the IC_{50} value of TROS, a dose-response experiment was performed with TROS concentrations ranging from 0.01 to 1 μM. TROS was preincubated for 30 min, and subsequently 1000 IU/ml TNF was applied. IL8 and TNF expression after 6 h was determined by qPCR, and IL8 levels in supernatant were determined with Bio-Plex after 24 h. Ctrl, Nb Alb-Ctrl-Ctrl, an irrelevant control Nanobody. *, 0.01 ≤ p < 0.05; **, 0.001 ≤ p < 0.01; ***, 0.001 ≤ p < 0.001; ****, p < 0.0001. The experiment was done in triplicate and data represent mean ± S.E. qPCR data were normalized to stable housekeeping genes.
bodies of Camelidae, in which they can be easily generated by immunization. These small recombinant molecules of 15 kDa offer many advantages over the generally used antibodies (Abs) or antibody fragments (Fab fragments) (20). To generate hTNFR1-specific Nbs, we immunized alpacas with hTNFR1 and subsequently identified eight different Nbs. These Nbs were produced in large quantities in the economical E. coli and P. pastoris expression systems (19). This stresses another important advantage of Nbs compared with the available anti-TNF therapies, which are mainly expensive biologicals that impose a heavy financial burden (45). Binding and inhibition experiments revealed that Nb 96 and Nb 70 have strong affinity for hTNFR1, but only Nb 70 effectively inhibited hTNFR1. Nb 96 and Nb 70 have different binding epitopes because only Nb 70 competed with TNF for hTNFR1 binding. This was confirmed with a predictive docking model, which showed that the epitope region of Nb 70 at least partially overlaps with the TNF-binding site, located mainly in CRD2 and CRD3 (46). Finally, when the two Nbs were simultaneously docked to hTNFR1, they bound different chains of the trimeric hTNFR1 without disturbing each other.

Nbs, encoded by a single exon, are very modular and can be combined to form multispecific and multivalent proteins (47). Consequently, we linked Nb 70 to Nb 96 with a flexible (G4-S)3 linker. Remarkably, modeling the binding of Nb 70 and Nb 96 to hTNFR1 revealed that the (G4-S)3 linker passes through the three chains of hTNFR1, which might hinder the interaction of TNF with TNFR1, and increases TNFR1 inhibition. Indeed, we demonstrated that the generation of TROS resulted in a 13-fold higher inhibitory capacity compared with the monovalent Nb 70.

In vitro, we further showed that TROS effectively inhibits TNF-induced gene expression of IL8, IκBα, and TNF as well as

FIGURE 9. A–F, inhibition of inflammation in human inflamed colonic biopsies. Inflamed colonic biopsies were obtained from patients suffering from active Crohn disease. Incubation with TROS for 24 h reduced the initial inflammatory expression of IL6 (A), IL8 (B), and TNF (C), in comparison with Nb Alb-Ctrl-Ctrl. This was also reflected in the supernatant, resulting in reduced IL6 (D), IL8 (E), and TNF (F) secretion. Nb Alb-Ctrl-Ctrl, an irrelevant control Nanobody. qPCR data were normalized to stable housekeeping genes. The connected lines represent data from the same patient. Data were analyzed using a paired t test.

FIGURE 10. A and B, inhibition of acute TNF-induced liver inflammation by TROS in humanized transgenic mice. TNF (6.7 mg/kg) was injected in uPA−/−,SCID mice with partially humanized liver (n = 9) pretreated with vehicle. This resulted in a significant increase of human IL6 expression in liver (A) and human IL6 levels (B) in serum after 2 h compared with mice injected with vehicle only (n = 6). Pretreatment with TROS (13.35 mg/ml) (n = 10) significantly antagonized inflammation in liver and serum. Data represent mean ± S.E., and statistical significances were calculated using an unpaired t test, comparing differences between vehicle only versus vehicle/TNF versus TROS/TNF. qPCR data were normalized to stable housekeeping genes.
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IL8 secretion in both a prophylactic as a therapeutic setup and in a dose-dependent manner with an IC_{50} of about 200 nm. Interestingly, TROS inhibited TNF-induced inflammation to the same extent as Etanercept. The generation of this trivalent Nb also improved binding kinetics because TROS had higher affinities than its monovalent equivalents.

The large size of conventional Abs (about 150 kDa) limits tissue penetration and binding to small receptor pockets, whereas Nbs show good tissue penetration. Conversely, the small size of monovalent Nbs limits their half-life to about 1.5 h (47). To overcome this limitation, we linked Nb 96 and Nb 70 to a Nb that binds serum albumin (48) to generate Nb Alb-70-96 (TROS). This extended the half-life to 24 and 30 h after i.v. and i.p. injection, respectively, which correlates with the half-life of murine serum albumin (1.07–1.6 days). Translation of the Nb in humans will further extend its half-life to about 19 days (49). The presence of the anti-albumin Nb could have another advantage, it might direct the Nb to regions of inflammation because albumin tends to accumulate there (50, 51).

Nbs are known to be very specific, and in vitro analysis excluded cross-reactivity with human TNFR2 and mouse TNFR1 because their extracellular parts share 28 and 71% sequence homology with that of human TNFR1 (2, 53). This might be a strong advantage of TROS compared with the available anti-TNF biological, which inhibits both TNFR1 and TNFR2 signaling. Indeed, it has been shown in the collagen-induced arthritis murine model of RA that disease severity is reduced in TNFR1-deficient mice (54), whereas TNF-driven arthritis is aggravated in TNFR2-deficient mice (55, 56). Selective TNFR1 inhibitor blocks inflammation by enabling T_{reg} to suppress IL17 production, and it promotes T_{reg} activity via TNFR2 signaling (57, 58). Also in CD, murine and human data point to an important role for T_{reg} in the suppressive functions of which are attributed to TNFR2 (59, 60). Indeed, in mice T_{reg} are critical for maintaining intestinal tolerance to luminal antigens and for preventing intestinal inflammation (61). Moreover, untreated CD patients have fewer or dysfunctional T_{reg} at the site of inflammation (61–63). Selective TNFR1 inhibition could ignore specific TNF/TNFR2 signaling to boost T_{reg} responses, and this might prove to be an effective new approach for patients not responding to anti-TNF treatment.

Interestingly, a HEK-293 blue cell assay revealed that TROS inhibits not only TNF/TNFR1 signaling but also LTA/TNFR1 signaling, whereas a commercially available hTNFR1 Ab does not. This property is again an advantage over the current anti-TNF therapies, because none of them, except Etanercept, bind LTA and block its signaling (64, 65). Additionally, papers suggest that LTA inhibition has value in the treatment of autoimmune diseases because LTA/TNFR1 signaling may lead to inflammation (66). The pro-inflammatory role of LTA has been shown in animal models of RA and multiple sclerosis (67, 68). For example, an RA patient was unresponsive to IFX anti-TNF therapy but switching to ETA, which also inhibits LTA, resulted in clinical remission, suggesting that in patients who are anti-TNF-resistant, it is not TNF but LTA signaling through TNFR1 that is the predominant cytokine (67).

In a more clinically relevant approach, acutely inflamed colon biopsies obtained from CD patients were cultured ex vivo and incubated with TROS. This led to reduction of the inflammatory signature compared with incubation with Nb Alb-Ctrl-Ctrl, as reflected in reduced expression of IL8, IL6, and TNF, which are known mediators of CD and are also considered as inflammation biomarkers. Interestingly, TNF is also considered the major inducer of IL8 and IL6 in RA, so TROS might be a valuable alternative to the available TNF antagonists (69, 70). Finally, we demonstrated in vivo the functionality of TROS during acute TNF toxicity in the human liver chimera uPA^{+/+}/SCID mouse model (32). We showed that intraperitoneal administration of TROS selectively antagonized the effects of TNF, as reflected in reduced expression of human IL8, ICAM, and VCAM. In agreement with this, human IL6 serum levels were also significantly reduced in the presence of TROS, further proving the inhibitory activity of TROS. In the long term, it will be important to compare the effects of TROS with the effects of other commercial and clinically used TNF antagonists, such as IFX, ADA, and ETA in vivo using humanized mouse models.

Importantly, even if TROS were to be less effective than or equally effective to the available TNF antagonists, it might overcome another of their disadvantages. Indeed, several CD or RA patients become unresponsive over time (6, 16, 71, 72) as follows: between 23 and 46% for IFX and ADA in CD patients and ~50% of RA patients receiving IFX (43, 73). In both diseases, secondary unresponsiveness is often associated with the development of anti-drug antibodies, because biological drugs may elicit immune reactions. Those anti-drug antibodies can nullify the therapeutic effect by neutralizing the drug or enhancing its clearance (74, 75). They might also cause fatal hypersensitivity reactions (76). Nbs can overcome this serious problem because they are suspected of being less immunogenic than conventional Abs, due to their high sequence homology with the human conventional VH framework regions (80–90%) and high conformational stability. By making a few amino acid substitutions in the framework region, they can be humanized even further (47, 77, 78).

Finally, a TNFR1-specific antagonist might also be useful for diseases in which anti-TNF treatment is ineffective or even exacerbates the disease, such as multiple sclerosis (79, 80). In a multiple sclerosis mouse model (experimental autoimmune encephalomyelitis), disease development was delayed in TNFR1 knock-out (KO) mice, in contrast to TNFR2 KO mice that developed a more severe and chronic disease (81, 82). Thus, again, TNFR1 was found to be responsible for the detrimental signals, although TNFR2 was essential for resolving inflammation and initiating repair (82). Furthermore, TNFR1 neutralization may indirectly stimulate TNF/TNFR2 signaling in the experimental autoimmune encephalomyelitis lesions, which subsequently promotes remyelination in chemically induced demyelination (83). In addition, type I diabetes and systemic lupus erythematosus might also depend on the immunomodulating TNF/TNFR2 signaling for protection or recovery (52, 84). Other diseases that can benefit from exclusive TNFR1 inhibition have been reviewed (3).

In conclusion, we describe for the first time the development of a trivalent Nanobody (Nb Alb-70-96, named TROS) that selectively binds and inhibits the TNF/TNFR1 signaling path-
way. TROS is strongly expressed in the economical P. pastoris expression system, has an acceptable half-life in mice, and has $K_d$ and $IC_{50}$ values in the nanomolar range. We show the robust inhibitory actions of TROS in vitro, ex vivo, and in vivo. In vitro, TROS reduced TNF-induced NF-$k$B gene expression in a dose-dependent manner. In ex vivo human colon biopsies, TROS reduced colon inflammation, and in an in vivo system of transgenic humanized mice, TROS antagonized inflammation in a model of acute TNF-induced shock.

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