ATROSAB, a humanized antagonistic anti-tumor necrosis factor receptor one-specific antibody

Kirstin A. Zettlitz,1 Verena Lorenz,2 Karlheinz Landauer,3 Sabine Münkel,1 Andreas Herrmann,2,3 Peter Scheurich,1 Klaus Pfizenmaier1 and Roland E. Kontermann1,*

1Institut für Zellbiologie und Immunologie; Universität Stuttgart; Stuttgart, Germany; 2Celonic GmbH; Jülich, Germany; 3Celonic AG; Basel, Switzerland

Key words: humanized IgG, antagonistic antibody, tumor necrosis factor receptor 1, epitope mapping

Abbreviations: ATROSAB, anti-TNF receptor one-specific antibody; TNF, tumor necrosis factor; TNFR1, TNF receptor 1

Tumor necrosis factor (TNF) signals through TNFR1 and TNFR2, two membrane receptors, and TNFR1 is known to be the major pathogenic mediator of chronic and acute inflammatory diseases. Present clinical intervention is based on neutralization of the ligand TNF. Selective inhibition of TNF receptor 1 (TNFR1) provides an alternative opportunity to neutralize the pro-inflammatory activity of TNF while maintaining the advantageous immunological responses mediated by TNFR2, including immune regulation, tissue homeostasis and neuroprotection. We recently humanized a mouse anti-human TNFR1 monoclonal antibody exhibiting TNFR1-neutralizing activity. This humanized antibody has been converted into an IgG1 molecule (ATROSAB) containing a modified Fc region previously demonstrated to have greatly reduced effector functions. Purified ATROSAB produced in CHO cells showed strong binding to human and rhesus TNFR1-Fc fusion protein and mouse embryonic fibroblasts transfected with a recombinant TNFR1 fusion protein with an affinity identical to the parental mouse antibody H398. Using chimeric human/mouse TNFR1 molecules, the epitope of ATROSAB was mapped to the N-terminal region (amino acid residues 1–70) comprising the first cysteine-rich domain (CRD1) and the A1 sub-domain of CRD2. In vitro, ATROSAB inhibited typical TNF-mediated responses like apoptosis induction and activation of NFκB-dependent gene expression such as IL-6 and IL-8 production. These findings open the way to further analyze the therapeutic activity of ATROSAB in relevant disease models in non-human primates.

Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine and a central mediator of inflammation. Elevated levels of TNF are associated with various inflammatory diseases including rheumatoid arthritis, psoriasis and Crohn disease. Several TNF-neutralizing reagents have been approved for the treatment of these diseases, including a soluble TNF receptor, etanercept, as well as the anti-TNF antibodies infliximab, adalimumab, certolizumab pegol and golimumab and many more are under development.1,2 With over 1 million patients treated with TNF antagonists, therapeutic efficacy is well-documented.3 However, global TNF inhibition over a prolonged period of time increases the risk of tuberculosis (TB) reactivation, serious infections and even malignancies.4–6 Consequently, medical information of all approved anti-TNF medicines includes extensive warnings and precautions.

Two TNF receptors (CD120a, TNFR1; CD120b, TNFR2) mediate signal transduction upon binding of TNF.7 Pro-inflammatory responses are mainly mediated by the ubiquitously expressed TNFR1. TNFR1 is activated both by the membrane-bound form of TNF (mTNF) and soluble TNF (sTNF), which is produced from mTNF by proteolytic cleavage. In contrast, TNFR2, expressed in a more restricted manner, e.g., by immune cells, endothelial cells and neurons, can only be activated by mTNF. Activation of TNFR2 mainly induces anti-apoptotic signals and can lead to cell proliferation in vitro.8 Furthermore, TNFR2 appears to play a role in tissue homeostasis and regeneration.9

Selective inhibition of TNFR1 signaling has gained increasing attention as an alternative to global TNF neutralization, which affects both TNF receptors. The application of receptor-selective rather than global inhibition of TNF responses represents a paradigm shift in the present clinical practice of treating inflammatory diseases. Conceptually, the selective inhibition strategy targets the predominant pathogenic pathway and potentially leaves signals essential for tissue homeostasis and immunocompetence untouched. This will in the long run improve therapeutic efficacy by minimizing therapy-limiting unwanted side effects, such as recurrence of TB and risk of malignancies or increased relapse rates in multiple sclerosis (MS),10 probably due to lack of TNFR2-mediated myelin regeneration.11 Recently, a TNF mutein (R1antTNF) selectively neutralizing the activity of TNFR1 has been described.12 This TNF mutein, administered either as
was used. ATROSAB was produced in mammalian cells and showed a similar binding and neutralizing behavior as the parental mouse H398 IgG. Furthermore, using chimeric human/mouse receptor molecules, we mapped the epitope of ATROSAB to the first 70 amino acids of human TNFR1.

Results

Production and binding activity of IZI-06.1 IgG (ATROSAB). The humanized anti-human TNFR1 antibody IZI-06.1 was converted into a human IgG1 using a heavy chain with greatly reduced effector functions (IgG1e3). This antibody (ATROSAB) was produced in CHO cells. A 25 L scale production of ATROSAB was performed in a wave system over a period of 15 days with a maximum cell density of more than 12 mio cells/mL. Purity and integrity was confirmed by SDS-PAGE analysis and size exclusion chromatography (Fig. 1A and B). ATROSAB showed strong binding to recombinant human TNFR1-Fc composed of the extracellular region of TNFR1 fused to the human IgG1 Fc region (Fig. 1C). The parental antibody, H398, exhibited an identical binding in ELISA. The selectivity for TNFR1 was confirmed by flow cytometric analysis of ATROSAB using mouse embryonic fibroblasts (MEF) transfected with fusion proteins comprising the extracellular domain of TNFR1 or TNFR2, respectively, fused to the intracellular domain of human Fas (TNFR1-Fas TNFR2-Fas). In this assay, binding was only seen with MEF-TNFR1-Fas, but not with MEF-TNFR2-Fas (Fig. 2A and B). Binding of ATROSAB to MEF-TNFR1-Fas was comparable to that of H398 as shown by a titration of antibody concentration (Fig. 2C). The EC50 values were approximately 0.1 nM for both ATROSAB and H398. Next, we investigated species specificity with recombinant mouse TNFR1-Fc and rhesus TNFR1-Fc fusion proteins. In ELISA, binding of the two antibodies was observed for human and rhesus TNFR1-Fc, but not mouse TNFR1-Fc (Fig. 3B).

Affinity measurements. The affinity of ATROSAB for TNFR1 was determined by quartz crystal microbalance measurements using immobilized TNFR1-Fc. ATROSAB bound with sub-nanomolar affinity to human and rhesus TNFR1-Fc, similar to the affinity of H398 for human TNFR1-Fc and rhesus TNFR1-Fc (Fig. 4 and Table 1). An approximately 10-fold reduced affinity was measured for monovalent scFv IZI-06.1, due to a faster off-rate, indicating that binding of ATROSAB and H398 to the dimeric TNFR1-Fc fusion proteins is influenced by avidity effects.

Antagonistic activity of ATROSAB. ATROSAB inhibited in a dose-dependent manner the TNF-induced apoptosis of Kyn-1 cells (Fig. 5). In this assay, a TNF concentration that resulted in 90% cytotoxicity was used. About half-maximal cytotoxicity, i.e., 55% viable cells, was observed at 60 nM for ATROSAB and 8 nM for H398, respectively. We then investigated the effects of ATROSAB on TNF-induced secretion of IL-6 from HeLa cells and IL-8 from HT1080 cells, respectively. TNF induced strong secretion of IL-6 from HeLa cells in a dose-dependent manner, reaching approximately 700 pg/ml of IL-6 after incubation with 4 nM TNF (200 ng/ml) for 18 h. Similarly, TNF induced

![Figure 1. Characterization of ATROSAB. (A) SDS-PAGE analysis of purified ATROSAB (4 μg/lane, Coomassie staining) analyzed under non-reducing (1) or reducing (2) conditions. (B) Size exclusion chromatography of ATROSAB (the position of standard proteins is indicated). (C) ELISA of ATROSAB and H398 for binding to human TNFR1-Fc.](image-url)
secretion of IL-8 from HT1080 cells reached approximately 7,000 pg/ml after incubation with 4 nM TNF for 18 h (Fig. 6C and D). ATROSAB and H398 inhibited release of IL-6 from HeLa cells and IL-8 from HT1080 cells induced by 20 pM TNF (1 ng/ml) in a dose dependent manner (Fig. 6A and B). In these assays, the IC_{50} values were 6 nM for ATROSAB and 6 nM for H398 for inhibition of IL-6 release (Fig. 6A) and for inhibition of IL-8 release (Fig. 6B), respectively. Incubation of HeLa cells or HT1080 cells (in the absence of TNF) with ATROSAB and H398, respectively, resulted in only marginal induction of cytokine release at a very narrow dose range. At concentrations around 10 nM only, slightly elevated IL-6 levels were observed (40–60 pg/ml vs. 15 pg/ml of untreated cells), corresponding to 3–4.5% of the response at a comparable TNF concentration (4 nM). For IL-8, the level was increased from 80 pg/ml of untreated cells to approximately 200 pg/ml after incubation with the antibodies, corresponding to approximately 2% of the equivalent TNF response. Human IgG included as negative control had no effect on cytokine release.

**Discussion**

Here, we describe the generation of an IgG1 derivative (ATROSAB) of a humanized TNFR1-specific antagonistic monoclonal antibody. The IgG format was chosen because of its long half-life, established production and increased binding due to bivalency. In order to avoid induction of antibody-mediated effector functions, ATROSAB possesses a mutated Fc-region previously described to exhibit greatly reduced ADCC and CDC. 21

Receptor-selective inhibition by ATROSAB and the parental mouse antibody resulted in blocking of distinct signaling pathways of TNFR1, as shown by inhibition of TNF-mediated cell death, as well as NFκB induced IL-6 and IL-8 release. Both cytokines are biomarkers of inflammation and are elevated, e.g., during episodes of active disease, in rheumatoid arthritis. The antagonistic activity of the murine H398 and the humanized monoclonal Fab was described to be based on interference with ligand binding. Using deletion mutants, the epitope recognized on TNFR1 was previously described to include the membrane-distal CRD1. By using a domain swapping strategy for chimeric mouse/human TNFR1-Fc fusion proteins, we now show that the epitope recognized by ATROSAB and H398 also
by steric hindrance or by inducing a conformational change, but could also interfere with homotypic PLAD interactions, thereby blocking the formation of functional TNFR signal complexes.

ATROSAB showed a slightly reduced antagonistic activity compared to H398. This is probably not due to altered affinity since affinities of both antibodies for recombinant human TNFR1 were similar as determined by quartz crystal microbalance measurements and in flow cytometry measurements using TNFR1-expressing cells. Currently, we cannot exclude that ATROSAB and H398 bind in a slightly different way or to a slightly different area within the identified region (aa 1–70) containing the epitope. Further epitope mapping by site directed mutagenesis of exposed residues will provide insights into the exact localization of the conformational epitope of ATROSAB and H398 and the mechanism of ligand blocking.

In the absence of TNF, for both antibodies (H398 and ATROSAB) a minor stimulatory activity was revealed at a very narrow dose range by sensitive in vitro assays with established cell lines. This marginal effect of the bivalent antibodies on the cytokine release might be caused by some cross-linking of receptors because, for monovalent Fab fragments of ATROSAB and H398 in the same assays, no stimulatory activity could be discerned over a 4-log dose range (data not shown). However, when compared with the cellular response to TNF treatment, this minor activity of bivalent antibodies appears negligible, amounting at peak levels to 2–5% of a genuine TNF response. Moreover, on freshly isolated human peripheral blood T cells and granulocytes, no agonistic activity of the TNFR1 specific antibodies could be discerned in the TNF-dependent cellular response models of T-cell activation and O$_2^-$ production, respectively (unpublished data).

Importantly, we could demonstrate binding of ATROSAB to rhesus TNF-R1 with a similar affinity as for human TNF-R1, thus allowing in vivo evaluation of ATROSAB in rhesus monkeys. The collagen-induced arthritis (CIA) model is the recognized standard for potential RA therapeutics and could be already reproducibly induced in rhesus macaques.29 Because of the well-established proximity (physiological, anatomical, genetic, microbiological and immunological) with humans, CIA in rhesus monkeys represents a very useful preclinical model for evaluation of safety and efficacy of novel therapies30 and enables the analysis of ATROSAB’s neutralizing activity and safety in non-human primates.

TNFR1-selective antagonists, such as ATROSAB, may become new therapeutic options for diseases against which currently marketed anti-TNF therapeutics have failed or even exacerbate disease progression, including multiple sclerosis, congestive heart failure, metabolic diseases (e.g., type II diabetes), cytokine release syndrome, septic shock, acute (e.g., stroke) and chronic (e.g., Alzheimer and Parkinson disease) neurodegenerative diseases. The potential application of TNFRI antagonists for central nervous system (CNS) diseases requires a thorough consideration of bioavailability. This point needs to be specifically addressed in further preclinical studies in appropriate animal disease models where the antibodies are crossreactive to the species TNFR1, such as baboon and rhesus monkeys or huTNFR1 k/i

includes subdomain A1 of CRD2, i.e., the entire epitope is covered by amino acids 1–70 in the N-terminal region of TNFR1. The finding that subdomain A1 of CRD2 is also required for antibody binding hints toward steric blockage as cause for neutralization of TNF action. The structure of TNFR1 with bound TNF (Fig. 9) shows that the identified epitope region at least partially overlaps with the TNF binding site, which is mainly located in CRD2 and CRD3.24 Additionally, site directed mutagenesis revealed that residues Pro23 and Gln24 of subdomain A1 of CRD1 directly contribute to antigen and species specificity. This is of interest because CRD1 is not directly involved in ligand binding,24 but is critically involved in TNFR1 signaling. CRD1 controls high affinity ligand binding by stabilizing the conformation of the subsequent CRD2,25 and removal of CRD1 results in loss of ligand binding.25-27 In addition, CRD1 comprises a homophilic receptor/receptor interaction site, the pre-ligand-binding assembly domain (PLAD), which is essential for generation of functional TNFR signal complexes.26,28 Hence, binding of ATROSAB to CRD1 could not only displace TNF

![Figure 3](image-url)
Materials and Methods

Materials. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Fc specific) antibody, HRP-conjugated anti-human IgG (whole molecule, Fc specific, Fab specific) antibodies, respectively, were purchased from Sigma (Taufkirchen, Germany). PE-labeled anti-mouse (whole molecule) and anti-human IgG (γ-chain specific) antibodies, respectively, were purchased from Sigma (Taufkirchen, Germany). Mouse embryonic fibroblasts (MEF) transfected with TNFR1-Fas (MEF-TNFR1-Fas) and TNFR2-Fas (MEF-TNFR2-Fas), respectively, were grown in RPMI 1640 medium, 5% FCS, 2 mM L-glutamine, 2 μg/ml puromycin. The human rhabdomyosarcoma cell line Kym-1 was mouse lines that are presently under development. Irrespective of this, we argue that clinical experience suggests that in several acute or chronic inflammatory CNS diseases, e.g., MS, the blood brain barrier is disturbed and can be leaky to various extents, thus enabling access of IgG molecules to the site of wanted action. ATROSAB could be an especially useful therapeutic alternative in diseases already known to respond to anti-TNF treatment and particularly in those diseases where specific blockage of TNFR1 and maintenance of TNFR2 function appears as a promising therapeutic approach.

![Image of diagrams](image-url)

**Figure 4.** Determination of affinity of H398 and ATROSAB for binding to human and rhesus TNFR1-Fc by quartz crystal microbalance (QCM) measurements. (A) Binding of H398 to human TNFR1-Fc, (B) binding of ATROSAB to human TNFR1-Fc, (C) binding of H398 to rhesus TNFR1-Fc and (D) binding of ATROSAB to rhesus TNFR1-Fc.

**Table 1.** Binding kinetics of H398 and ATROSAB

| Antibody   | Antigen     | $R_{\text{max}}$ (Hz) | $k_{\text{on}}$ (M⁻¹s⁻¹) | $k_{\text{off}}$ (s⁻¹) | $K_D$ (M)  |
|------------|-------------|-----------------------|--------------------------|-----------------------|------------|
| H398       | huTNFR1-Fc  | 45.4                  | $3.1 \times 10^5$        | $7.0 \times 10^{-5}$   | $2.3 \times 10^{-10}$ |
| H398       | rheTNFR1-Fc | 30.4                  | $2.1 \times 10^5$        | $1.0 \times 10^{-4}$   | $4.9 \times 10^{-10}$ |
| ATROSAB    | huTNFR1-Fc  | 46.6                  | $3.8 \times 10^5$        | $1.3 \times 10^{-4}$   | $3.5 \times 10^{-10}$ |
| ATROSAB    | rheTNFR1-Fc | 34.9                  | $6.9 \times 10^5$        | $6.7 \times 10^{-5}$   | $1.0 \times 10^{-10}$ |
| scFv IZI-06.1 | huTNFR1-Fc | 7.2                   | $3.5 \times 10^6$        | $7.6 \times 10^{-4}$   | $2.2 \times 10^{-9}$  |
grown in RPMI 1640 medium, 10% FCS, 2 mM L-glutamine and HT1080wt cells and HeLa cells were grown in RPMI 1640 medium, 5% FCS, 2 mM L-glutamine.

Production of IZI-06.1 IgG (ATROSAB). DNA encoding the light and heavy chain of ATROSAB, including Igκ signal sequences and codon-optimized for production in CHO cells, was produced synthetically (Geneart, Regensburg, Germany). The light chain (LC) DNA was cloned as BamHI/NotI fragment into shuttle vector pCV072 (Celonic GmbH, Jülich, Germany) and the heavy chain (HC) DNA was cloned as KasI/NheI fragment into pFUSE (InVivoGen, Toulouse, France). pFUSE-HC was digested with SmaI (Swal) and the resulting blunt end fragment containing the entire HC expression cassette was cloned into pCV072-LC digested with PsiI. In this bicistronic expression cassette, the light chain is under the control of the P_{CMV enhomed} promoter and the heavy chain gene is controlled by the P_{CMV enhomed} promoter. The stably transfected CHO cells were grown in CDM4PermAB (Thermo Fischer, Erembodegem, Belgium) and cultivated in fed-batch mode in a 25 L wave bioreactor system (Sartorius Stedim, Melsungen, Germany) with a soy hydrolisate feeding solution (Kerry Biosciences, Almere, The Netherlands). Antibody was purified from cell culture supernatant by using Protein A chromatography (GE Healthcare, Uppsala, Sweden), followed by a membrane intermediate step with Sartobind Q single Sep mini (Sartorius Stedim. Melsungen, Germany). Final product was obtained via a buffer exchange step.

Production of TNFR1-Fc fusion proteins. DNA encoding the extracellular region of human TNFR1 (aa 29–211), rhesus TNFR1 (aa 27–209) and mouse TNFR1 (aa 30–212) was produced synthetically (Geneart, Regensburg, Germany), introducing appropriate restriction sites between the individual domains and cloned into pSecTagL1-Fc (modified from pSecTag-FcHis). Chimeric human/mouse TNFR1-Fc fusion proteins were generated by exchanging the different regions between human and mouse TNFR1-Fc. HEK293 cells were transfected with plasmid DNA using lipofectamine (Invitrogen, Karlsruhe, Germany) and stably transfected clones were selected in the presence of zeocin as described. Cells were expanded in RPMI, 5% FCS, 2 mM L-glutamine to 90% confluence. For protein production, the medium was substituted with Opti-MEM I (Invitrogen, Karlsruhe, Germany) and supernatant was collected every 3–4 days. Proteins were purified from cell culture supernatant by protein A chromatography. In brief, supernatants were adjusted to pH 8 by adding 1/10 volume of 1 M TrisHCl, pH 8.0 and loaded onto a protein A-Sepharose CL-4B column (Sigma, Taufkirchen, Germany). Bound protein was eluted with 100 mM glycine pH 3.0, neutralized by adding 1/10 volume 1 M TrisHCl, pH 8.0 and protein containing fractions were dialyzed against PBS. Protein concentrations were determined photometrically and purity was analyzed by SDS-PAGE and immunoblotting using an HRP-conjugated anti IgG (Fc specific) antibody (Sigma, Taufkirchen, Germany).

**Protein characterization.** Size exclusion chromatography (SEC) was performed by HPLC using a BioSuite™ 250, 5 μm HR SEC (Waters GmbH, Eschborn, Germany). The following standard proteins were used: apoferritin (44 kDa), β-amylase (200 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), aprotinin (6.5 kDa).

**Affinity measurements.** Affinities of the antibodies were determined by quartz crystal microbalance measurements (QCM; Attana A100, C-Fast system, Stockholm, Sweden). Binding experiments were performed in PBS 0.005% Tween 20 at a flow rate of 25 to 35 μl/min and temperature was controlled at 20°C. The TNFR1-Fc fusion proteins were chemically immobilized on an Attana carboxyl sensor chip by amine coupling at a concentration of 50 μg/ml according to the manufacturer’s protocol resulting in a signal increase (frequency shift) of approximately 200 Hz. Antibodies were analyzed at concentration between 62.5 and 3.9 nM (4 measurements per concentration). The chip was regenerated with 10 mM glycine-HCl, pH 3.0. Buffer injections were performed prior to each sample injection to use as a reference in Attester Evaluation. Data were collected by Attesting 3.0 (Version 3.1.1.8, Attana, Stockholm, Sweden) and analyzed by ClampXP. A mass transport model was fitted to the data.

**ELISA.** Recombinant human TNFR1-Fc fusion protein was immobilized in 96-well plates (50 ng/well in PBS) overnight at 4°C. After 2 h blocking with 2% (w/v) dry milk/PBS, recombinant antibody fragments were titrated in duplicates and incubated for 1 h at RT. Detection was performed with HRP-conjugated anti-human IgG (Fab-specific) antibody and HRP-conjugated anti-mouse IgG (Fc-specific) using TMB substrate (1 mg/ml TMB, sodium acetate buffer pH 6.0, 0.006% H₂O₂). The reaction was stopped with 50 μl of 1 M H₂SO₄. Absorbance was measured at 450 nm in an ELISA-reader.

**Flow cytometry.** Binding to TNFR1-Fas or TNFR2-Fas transfected MEF cells was analyzed by flow cytometry. Cells (2 x 10⁶) were incubated with dilution series of antibodies for 4 h at 4°C. Cells were then washed with PBS and bound antibodies were detected with PE-labeled goat anti-mouse or anti-human antibody. Cells were analyzed by flow cytometry (Cytomics FC 500, Beckmann-Coulter, Krefeld, Germany). Data were

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**Figure 5.** Inhibition of TNF-mediated cytotoxicity (1.25 ng/ml TNF) on Kym-1 cells by ATROSAB and H398. Cells were analyzed after 6 h by crystal violet staining (n = 3). Maximum (10% viability of control) and half maximum (55% viability of control) are displayed in dotted lines.
evaluated with the program WinMDI, version 2.9 and fitted with GraphPrism software (La Jolla, CA, USA) from three independent binding curves.

Cytotoxicity. Kym-1 cells (1.5 x 10^4 cells/100 μl) were grown in 96-well plates overnight. A constant amount of human soluble TNF (1.25 ng/ml in medium) was applied after pre-incubation with antibodies in triplicates (concentrations as indicated in the figures) in medium for 1 h. After 7 h, cells were stained by crystal violet (20% methanol, 0.5% crystal violet) for 15 min. The wells were washed with H_2O and air-dried. The dye was resolved with methanol for 15 min and optical density at 550 nm was determined (Tecan infinite M200, Crailsheim, Germany).

IL-6 and IL-8 assays. HT1080 cells (2.0 x 10^5 cells/100 μl) were grown in 96-well plates overnight. The next day, the medium was exchanged to remove constitutively produced IL-8 and the cells were incubated in duplicates together with serial dilutions of human soluble TNF for additional 18 h. Induction of IL-8 production and secretion into the culture supernatant was determined by an IL-8-Sandwich ELISA (ImmunoTools, Friesoythe, Germany) according to the manufacturer’s protocol. In addition,

Figure 6. Inhibition of IL-6 and IL-8 secretion induced by TNF by ATROSAB and H398. HeLa cells (A) or HT1080 cells (B) were incubated with TNF (1 ng/ml) and increasing concentrations of ATROSAB or H398 and cytokine secretion were determined by ELISA (n = 3). Human IgG (hulG) was included as negative control. In the same way, effects of antibodies on cytokine secretion in the absence of TNF were determined. Compared with TNF, both antibodies had only marginal effects on IL-6 (C) and IL-8 (D) secretion.

Figure 7. Plasma half-lives of ATROSAB and H398 after a single dose i.v. injection (25 μg) into CD1 mice. Serum concentrations of antibodies were determined by ELISA.
cells were incubated with serial dilutions of antibodies in the absence and presence of TNF (1 ng/ml) and analyzed for IL-8 secretion after 18 h of incubation. In the same way, we analyzed the inhibitory effects of the antibodies on TNF-mediated secretion of IL-6 from HeLa cells using an IL-6 sandwich ELISA (ImmunoTools, Friesoythe, Germany) according to the manufacturer’s protocol.

**Plasma half-life.** CD1 mice (18–22 weeks, weight between 34 and 43 g) received an intravenous injection of 25 μg purified antibody (ATROSAB, H398) in a total volume of 150 μl. Blood samples (50 μl) were taken at 3 min, 1, 2, 4 and 7 days from the tail and incubated on ice for 15 min. Clotted blood was centrifuged at 13,000 g for 10 min and serum samples stored at -20°C. Serum concentrations were then determined by ELISA as described above. Terminal half-lives were calculated using concentrations at day 1 to 7.

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**Figure 8.** (A) Epitope mapping of ATROSAB and H398 using wild-type and chimeric human/mouse TNFR1-Fc fusion proteins. Antibodies (0.1 nM) were analyzed by ELISA for binding to the TNFR1-Fc fusion proteins. His-tagged human TNF (huTNF) was included as control. (B) Sequence comparison of the identified epitope region (aa 1–70) of human (huTNFR1), mouse (moTNFR1) and rhesus (rhTNFR1) TNFR1. Cysteine residues are marked with grey boxes and the two positions (P23, Q24) analyzed by site-directed mutagenesis are marked by asterisks.

**Figure 9.** (A) Structure of TNF (red) bound to TNFR1 (blue). The identified epitope region is marked in green. (B) A single TNFR1 chain. The two positions (P23, Q24) identified by mutagenesis to contribute to binding of ATROSAB and H398 are highlighted in dark green.
This work was supported by a grant from EC FP6, Project NeuproMiSe, contract # LSHM-CT-2005-018637.

Acknowledgements

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