Improved monovalent TNF receptor 1-selective inhibitor with novel heterodimerizing Fc

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ABSTRACT
The development of alternative therapeutic strategies to tumor necrosis factor (TNF)-blocking antibodies for the treatment of inflammatory diseases has generated increasing interest. In particular, selective inhibition of TNF receptor 1 (TNFR1) promises a more precise intervention, tackling only the pro-inflammatory responses mediated by TNF while leaving regenerative and pro-survival signals transduced by TNFR2 untouched. We recently generated a monovalent anti-TNFR1 antibody fragment (Fab 13.7) as an efficient inhibitor of TNFR1. To improve the pharmacokinetic properties of Fab 13.7, the variable domains of the heavy and light chains were fused to the N-termini of newly generated heterodimerizing Fc chains. This novel Fc heterodimerization technology, designated “Fc-one/kappa” (Fc1k) is based on interspersed constant Ig domains substituting the CH3 domains of a γ1 Fc. The interspersed immunoglobulin (Ig) domains originate from the per se heterodimerizing constant CH1 and CLκ domains and contain sequence stretches of an IgG1 CH3 domain, destined to enable interaction with the neonatal Fc receptor, and thus promote extended serum half-life. The resulting monovalent Fv-Fc1k fusion protein (Atrosimab) retained strong binding to TNFR1 as determined by enzyme-linked immunosorbent assay and quartz crystal microbalance, and potently inhibited TNF-induced activation of TNFR1. Atrosimab lacks agonistic activity for TNFR1 on its own and in the presence of anti-human IgG antibodies and displays clearly improved pharmacokinetic properties.

Introduction
Tumor necrosis factor (TNF) plays a central role in the immune system, especially during inflammation and infection. TNF activates the immune system and is mainly expressed by macrophages, triggered, for example, by lipopolysaccharides (LPS) from microbial sources. However, prolonged exposure to endogenous levels of TNF during inflammatory reactions reduces the responsiveness of immune cells and thereby attenuates the immune system. Thus, TNF is an important regulator of the immune system that requires strict temporal and spatial regulation in order to maintain immunological responsiveness and to prevent autoimmunity. Dysregulated expression of TNF is associated with severe inflammatory conditions and has been reported to be involved in the development of diseases such as systemic lupus erythematosus, type II diabetes, Crohn’s disease and rheumatoid arthritis (RA). TNF overexpression in RA was demonstrated to induce the expression of further pro-inflammatory cytokines, to promote vascularization and to support the influx of immune cells, resulting in manifestation of chronic inflammation and tissue damage.

Five TNF-targeting therapeutics (infliximab, adalimumab, certolizumab pegol, golimumab, etanercept) are presently approved and used for the treatment of different inflammatory diseases, resulting in dramatic improvement of the patients’ quality of life, for example, as assessed for inflammatory bowel disease. However, the undisputable clinical success of anti-TNF therapeutics has limitations. Depending on the applied anti-TNF agent, 13–33% of treated patients do not respond to treatment and up 46% stop to respond to therapy, resulting in either discontinuation or dose increase. Moreover, anti-TNF therapy can be associated with severe side-effects, including opportunistic infections, tuberculosis reactivation, development of malignancies (e.g., lymphomas) and neurological conditions (demyelinating disease), and the exacerbation of existing inflammatory symptoms (for reviews, see refs.19,20). Some side effects might be due to sustained and long-lasting blockade of TNF-mediated activation of its two distinct TNF receptors (TNFR1 and TNFR2), which have different or even opposing functions. While TNFR1 is mainly responsible for the induction of pro-inflammatory or, under certain conditions, apoptosis-inducing signals, TNFR2 is involved in cellular regeneration, tissue homeostasis, and immune attenuation. Therefore, selective inhibition of TNFR1 under inflammatory conditions represents a more specific intervention in TNF biology and promises reduced side effects due to unaffected TNFR2 signaling.

In preclinical disease models, TNFR1-blocking agents have already proved to be effective. For example, a commercially
available anti-mouse TNFR1 antibody reduced experimental autoimmune encephalomyelitis (EAE) symptoms27 and a chemically modified TNFR1-specific TNF mutant (PEG-R1ant-TNF) showed therapeutic efficacy in murine models of hepatitis, collagen-induced arthritis, arterial inflammation and intimal hyperplasia.28–30 Furthermore, a domain antibody (dAb) directed against TNFR1 (GSK1995057) attenuated disease severity in a murine model of acute respiratory distress syndrome,31 and a fusion protein composed of three different dAbs, two directed against different epitopes on TNFRI and one dAb directed against serum albumin (intended to extend the serum circulation of the therapeutic), delayed the onset of EAE and reduced typical disease symptoms.32 Importantly, cross-linking of TNFR1, which occurs, for example, by bivalent binding of the molecules or by secondary events like interactions with Fcγ receptors or anti-drug antibodies (ADA), must be avoided to maintain the antagonistic nature of the presently available TNFR1 inhibitors.

We recently converted a humanized anti-TNFR1 IgG1 antibody (Atrosab) into an affinity and stability improved monovalent antigen-binding fragment (Fab 13.7).33 Fab13.7 efficiently inhibited TNF-mediated activation of TNFRI. Moreover, this Fab completely lacked intrinsic agonistic activity, which was observed to a limited extent for the IgG Atrosab at a narrow concentration range due to its bivalent molecular structure. In order to improve the half-life of the Fab, various half-life extension strategies were applied, including the fusion of the Fv domains to a knobs-into-holes Fc region.33

In this study, we applied a newly developed Fc heterodimerization strategy to generate a novel monovalent Fv-Fc fusion protein. The novel Fc heterodimerization technology, designated “Fc-one/kappa” (Fc1κ) is based on interspersed immunoglobulin (Ig) domains, derived from the per se heterodimerizing constant Ig domains CH1 and CLκ, containing CH3 sequence sections, intended to mediate neonatal Fc receptor (FcRn) binding. The concept of the monovalent, antibody-derived Fv-Fc1κ fusion protein and its molecular composition compared to the natural TNFR1 ligand TNF, bivalent IgG and the monovalent Fab is depicted in Figure 1, highlighting the benefits of monovalency in combination with prolonged serum half-life.

**Results**

**Development of a novel heterodimerization module**

Novel heterodimerizing Ig domains were generated based on the first constant domain of the IgG1 heavy chain (CH1) and the constant domain of the κ light chain (CLκ, Figure 2). In order to enable binding to FcRn, amino acid sequence stretches of the third constant domain of the IgG1 heavy chain (CH3) that do not interfere with the CH3-CH3 interdomain interaction were transferred to the CH1 and CLκ domains in silico. This process resulted in two novel interspersed Ig domains. The first domain, CH31 contains amino acid sequence fragments of CH1 and CH3 and the second domain, CH3kappa (CH3κ) contains amino acid sequence fragments of CLκ and CH3. Furthermore, IgG1 CH2 domains were fused to the N-termini of CH31 and CH3κ, in order to provide the entire FcRn binding region of the IgG molecule.34 Finally, by the addition of IgG1 hinge region to the N-termini of the CH2 domains, we generated a novel covalently linked heterodimerizing Fc moiety, designated Fc-one/kappa (Fc1κ, Figure 2).

In order to evaluate the degree of heterodimerization, mediated by the newly generated Fc1κ chains, asymmetric single-chain variable fragment (scFv)-Fc1κ fusion proteins were generated by fusing an scFv moiety to the N-termini of either the CH31- or CH3κ-containing chains of Fc1κ (Figure 3). Moreover, similar molecules were cloned and produced, containing the state-of-the-art heterodimerizing “knobs-into-holes” Fc,35 wherein amino acid residues of the CH3–CH3 interface were exchanged by amino acids with larger (“knobs”-chain) or smaller side chains (“holes”-chain). Finally, a construct containing a wild-type CH3 on both chains was included in the set of analyzed molecules. All proteins (see Figure 3) were produced in transiently transfected HEK293E cells and purified by protein A affinity chromatography. SDS-PAGE analysis under reducing conditions demonstrated successful expression of both chains of the heterodimeric fusion proteins, except for the Fc1κ fusion protein carrying the scFv at the CH3κ-containing chain, which expressed the scFv-CH3κ chain, but expression of the CH2-CH31 chain could not be detected. Under non-reducing conditions, the scFv-Fc1κ fusion protein containing the scFv moiety at the

**Figure 1. Inhibition and activation of TNFR1.** TNFR1 is strongly activated by its natural, trimeric ligand TNF. The bivalent IgG ATROSAB was shown to exert a dominant antagonistic activity in the presence of TNF, yet on its own exerts a marginal TNFR1 activation in a narrow dose range. Monovalent formats like the Fab or the newly developed Fv-Fc1κ are effective antagonists of TNF mediated TNFR1 activation and lack intrinsic agonistic activity. In addition, the Fv-Fc1κ comprises an Fc proportion, providing a prolonged serum half-life, comparable to an IgG.
CH31 chain of the Fc1κ region and both proteins based on the knobs-into-holes technology revealed one clearly dominating band (89 kDa for scFv-CH31-CH3κ; 83 kDa for the two knobs-into-holes fusion proteins), demonstrating correct formation of the heterodimeric proteins with an expected molecular mass of 76 kDa and 77 kDa, respectively. Interestingly, the knobs-into-holes-based fusion protein, carrying the scFv moiety at the “knobs” chain showed an additional faint band around 130 kDa, indicating partial homodimerization of the heavy chain (scFv-Fc3knots). Furthermore, the Fc1κ fusion protein carrying the scFv at the CH3κ-containing chain, showed three bands, indicating the presence of covalently and non-covalently linked homodimers, as well as heterodimers, in the preparation. This observation indicates a residual propensity of the CH3κ-containing chain to form homodimers. Similarly, the fusion protein containing the wild-type Fc also showed three bands, indicating the formation of light chain and heavy chain homodimers and light chain/heavy chain heterodimers. Taken together, the generated interspersed Ig domains CH31 and CH3κ mediated heterodimerization of Fc chains in a configuration containing an scFv moiety connected to the

Figure 2. Generation of a heterodimerizing Fc with FcRn-interacting regions. A novel heterodimerizing Fc was generated by replacing the IgG1 CH3 domains with interspersed constant Ig domains, based on the per se heterodimerizing constant Ig domains CH1 and CLκ, complemented with elements of IgG1 CH3 domains, that are responsible for the interaction with the FcRn. The newly generated heterodimerizing Fc was designated Fc-one/kappa (Fc1k).

Figure 3. Proof of concept of Fc1k using asymmetric scFv-Fc1k fusion proteins. An scFv fragment was fused to the N-terminus of the hinge region of an Fc chain, containing either the newly generated CH31 or CH3κ domains, the heterodimerizing CH3knobs or CH3holes domains, or the wild type CH3 domains. Proteins were analyzed by SDS-PAGE (NuPAGE™ 4–12% Bis-TRIS Midi Gel). Descriptions: M (Marker), 1: (scFv connected to Hinge-CH2-CH31); 2: (scFv connected to Hinge-CH2-CH3knots); 3: (scFv connected to Hinge-CH2-CH3holes); 4: (scFv connected to Hinge-CH2-CH3knots); 5: (scFv connected to Hinge-CH2-CH3wt). Staining: Coomassie Brilliant Blue; de-staining: Water.
N-terminus of the CH31-containing Fc chain in a manner that was similar or even superior to the established knobs-into-holes technology.

**Generation of a monovalent TNFR1-specific antagonist**

The variable domains of the recently developed TNFR1-specific Fab 13.7 were fused to the CH2 domain N-termini of the CH31- or CH3κ-containing Fc chains via a short peptide linker, either fusing the VH to the CH2-CH31 chain and VL to the CH2-CH3κ chain (VH13.7-CH2-CH31 and VL13.7-CH2-CH2 2k; VH1C/VLkC, Figure 4(a)), or fusing VH to the CH2-CH3k chain and VL to the CH2-CH31 chain (VL13.7-CH2-CH31 and VH13.7-CH2-CH3κ; VL1C/VHkC; Figure 4(b)). After production in HEK293E cells by transient transfection and protein A affinity chromatography purification, both molecules revealed two bands of 38 kDa and 43 kDa in SDS-PAGE under reducing conditions (Figure 4(c, d)). Moreover, under non-reducing conditions both proteins showed a band of 70 kDa, indicating correct assembly and covalent connection of the dimeric protein. However, in the VH1C/VLkC configuration, there was an additional band visible at a size of 38 kDa under non-reducing conditions, indicating the presence of non-covalently linked or monomeric chains (Figure 4(c)). Finally, VH1C/VLkC and VL1C/VHkC both showed one major peak in size-exclusion chromatography (SEC) with an apparent molecular mass of 78 kDa (calculated 72 kDa) and a Stokes radius of 3.5 nm, corresponding to the assembled heterodimeric protein (Figure 4(c, f)). Consistent with the SDS-PAGE analysis, the molecule with the VH1C/VLkC configuration showed additional peaks, confirming the existence of free unligated monomeric chains. A small fraction of aggregated or multimerized protein could also be discerned. In conclusion, the VL1C/VHkC configuration was selected for further development steps of this improved TNFR1-specific antagonistic antibody-derived molecule, which will be referred to below as Atrosimab.

**Biochemical characterization of Atrosimab**

Atrosimab was produced in Chinese hamster ovary (CHO) cells after lentiviral transfection and purified by protein A affinity chromatography. The product was further purified by preparative SEC in order to remove oligomeric protein species. The final preparation revealed a single peak in SEC analysis with an apparent molecular mass of 81 kDa (72 kDa calculated) and a Stokes radius of 3.5 nm (Figure 5(a)). Two bands in SDS-PAGE under reducing conditions of 38 and 43 kDa and one band under non-reducing conditions of ~70 kDa confirmed purity and correct assembly of Atrosimab (Figure 5(b)). Mass spectrum analysis of deglycosylated Atrosimab further substantiated the correct formation of the heterodimer in the presence of negligible amounts of homodimerically assembled protein species (Fig. S1). Moreover, an aggregation point (melting temperature) of 64°C was determined by dynamic light scattering (Figure 5(c)). Finally, Atrosimab maintained full binding activity after incubation in human plasma for up to seven days, indicating good plasma stability for the analyzed period of time (Figure 5(d)).

**Binding of Atrosimab to TNFR1, C1q and Fcy receptors**

Atrosimab bound to human TNFR1-Fc in an enzyme-linked immunosorbent assay (ELISA) with an EC$_{50}$ value of 0.37 nM, representing a 2.2-fold reduction in binding activity compared to the parental Fab 13.7 (Figure 6(a)). The bivalent IgG antibody ATROSAB bound to TNFR1 with an EC$_{50}$ value of 0.09 nM. Furthermore, in real-time binding analyses using the quartz crystal microbalance (QCM, Figure 6(b)) technology, Atrosimab revealed a K$_D$ value of 2.66 nM with an association rate constant k$_o$ and a dissociation rate constant k$_{off}$ of $3.69 \times 10^{-5}$ M$^{-1}$s$^{-1}$ and $9.83 \times 10^{-4}$ s$^{-1}$, respectively.

The inability of Atrosimab to mediate Fc effector functions due to the Δab mutations that were introduced into Fc1κ was demonstrated by analyzing the ability of the complement protein C1q and the Fcy receptors Iα, IIb, and IIIa to bind to immobilized Atrosimab. Similar to the previously published antagonistic TNFR1-specific IgG ATROSAB, C1q and the Fcy receptors Iα, IIb, and IIIa revealed strongly reduced or absent binding to Atrosimab compared to the control antibody Rituximab, which possesses a wild-type IgG1 Fc (Figure 5(c)).

**In vitro bioactivity of Atrosimab**

Atrosimab demonstrated a complete lack of agonistic activity over a concentration range from 50 pM to 500 nM, as demonstrated in interleukin-6 (IL-6) and interleukin-8 (IL-8) release assays using HeLa and HT1080 cells, respectively, and in a cell death induction assay using Kym-1 cells (Figure 7(a-c)). As expected, the parental Fab 13.7, used as monovalent control protein due to its previously demonstrated absence of agonism, did not activate TNFR1. In contrast, the previously described presence of a marginal agonistic activity at a narrow dose range of the bivalent humanized antibody ATROSAB was confirmed in IL-6 and IL-8 release experiments (Figure 7(a, b)). However, this minor induction of TNFR1 activation was not detectable in the cell death induction assay using Kym-1 cells (Figure 7(c)).

Atrosimab inhibited the activation of TNFR1 in the presence of a constant concentration of TNF in IL-6 (0.1 nM TNF) and IL-8 release (0.1 nM TNF), as well as in cell death induction assays (0.01 nM TNF) with IC$_{50}$ values of 54.5 nM, 24.2 nM and 16.2 nM, respectively (Figure 7(d-f), Table 1). The corresponding IC$_{50}$ values of the parental Fab 13.7 in IL-6 release, IL-8 release and in cell death induction assays were 37.1 nM, 12.7 nM, and 9.5 nM, respectively, indicating a slightly reduced bioactivity of Atrosimab (1.5- to 1.9-fold). However, in comparison to ATROSAB, the antagonistic activity of Atrosimab was three- to fourfold increased, depending on the assay system (IL-6: 3.0; IL-8: 3.5; cell death: 4.0) (Figure 7(d-f), Table 1).

**Antibody-mediated cross-linking of Atrosimab**

In order to assess the risk of drug-specific antibodies to turn Atrosimab into a potentially agonistic molecule by secondary cross-linking, three different goat anti-human IgG serum preparations were analyzed for their potential to induce TNFR1 activation in combination with Atrosimab. In IL-8 release experiments, a constant concentration of anti-human IgG serum was incubated together with increasing concentrations...
Figure 4. Generation and optimization of Atrosimab. Two versions of a novel Fv-Fc1k fusion protein were generated, (a) composed of the chains VH-CH2-CH31 (VH1C)/VL-CH2-CH3k (VLkC) or (b) composed of VL-CH2-CH31 (VL1C)/VH-CH2-CH3k (VHkC). Both molecules were compared in SDS-PAGE (c and d, NuPAGE™ 4–12% Bis-TRIS Midi Gel) under reducing (R) and non-reducing conditions (NR) and SEC (e and f, Phenomenex Yarra SEC-2000, 300 × 7.8 mm, flow rate of 0.5 ml/min, mobile phase Na2HPO4/NaH2PO4).

of Atrosimab and the control proteins Fab13.7 and ATROSAB (Figure 8). Atrosimab and the monovalent control protein Fab13.7 did not induce release of IL-8 in combination with any of the tested sera, while the stimulation of HT1080 cells by the bivalent control protein ATROSAB in combination with all three anti-human IgG sera resulted in three- to
fivefold increased induction of IL-8 release at the maximum of the respective dose response (Figure 8(a–c); ATROSAB alone see Figure 7(b)). Binding of all three used anti-human IgG sera to Atrosimab and the control proteins was confirmed by ELISA (Fig. S2a–c). Moreover, Atrosimab and Fab13.7 revealed only slightly reduced binding to TNFR1-Fc in presence of all three anti-human IgG sera, as determined by ELISA (Fig. S2 d–f). In contrast, TNFR1 binding of ATROSAB was strongly reduced in the presence of anti-human Ig, which, however, did not prevent crosslinking-mediated TNFR1 activation, as demonstrated above.

**Pharmacokinetic analysis of Atrosimab**

To evaluate the effect of the novel heterodimeric Fc on *in vivo* serum circulation, the pharmacokinetic (PK) properties of Atrosimab were analyzed after bolus injection of 400 µg using C57BL/6J-huTNFRSF1A<sub>cd</sub>tm1UEG/izi mice carrying the gene
encoding the extracellular domain of the human TNFR1 at the respective mouse locus (Figure 9, Table 2). Atrosimab revealed initial and terminal half-lives of 2.2 ± 1.2 h and 41.7 ± 18.1 h, respectively, and an area under the curve of 5856.0 ± 1369.9 µg/ml × h.

**Discussion**

The objective of this study was to develop a novel, monovalent inhibitor of TNFR1 activation with extended serum half-life based on the previously described antagonistic TNFR1-specific Fab 13.7. To incorporate an IgG-like Fc into the molecule while retaining Fab-like heterodimerization of the polypeptide chains, the variable domains of Fab 13.7 were fused to the N-termini of newly generated heterodimerizing Fc chains, designated Fc-one/kappa (Fc1κ). Heterodimerization of this novel Fc moiety is mediated by interspersed Ig domains called CH31 or

**Table 1. Bioactivity of Atrosimab.**

|                      | Atrosimab | Fab 13.7 | ATROSAB |
|----------------------|-----------|----------|---------|
| IC50, IL-6 [nM]      | 54.5      | 37.1     | 164.7   |
| IC50, IL-8 [nM]      | 24.2      | 12.7     | 84.1    |
| IC50, Cell death induction [nM] | 16.2 | 9.5 | 64.4 |

**Figure 7. Antagonistic bioactivity of Atrosimab and lack of agonism.** The inherent lack of agonistic activity of Atrosimab in terms of TNFR1 activation was demonstrated in three individual assays: a) IL-6 release from HeLa cells, b) IL-8 release from HT1080 cells and c) cell death induction assay using Kym-1 cells. The inhibitory potential of Atrosimab was shown in an IL-6 release assay using HeLa cells (d), in an IL-8 release assay using HT1080 cells (e) and in a cell death induction assay using Kym-1 cells (f), which were performed in the presence of a constant concentration of 0.1 nM TNF (d and e) or 0.01 nM TNF (f). ATROSAB (marginal activity) and TNF (strong activity) alone served as control molecules for the activation of TNFR1. Fab 13.7 served as negative control (a, b and c). Fab 13.7 and ATROSAB served as controls for the inhibition of TNF-induced TNFR1 activation (d, e and f). All graphs represent the mean of three individual experiments, error bars indicate SD.

**Figure 8. Complete lack of agonistic bioactivity of Atrosimab in the presence of anti-human IgG antibodies.** The activation of TNFR1 on the surface of HT1080 cells by Atrosimab in the presence of a constant concentration (ca. 15.8 nM) of three different anti-human IgG serum preparations (a, b and c) was determined by the detection of IL-8 release into the culture supernatant. Unstimulated cells and 33 nM TNF were used as controls. The agonistic effect of potentially crosslinking antibodies was compared to Fab 13.7 and ATROSAB. All experiments show Mean ± SD of three individual experiments.
CH3κ. These interspersed Ig domains are composed of sequences originating mainly from the per se heterodimerizing IgG1 CH1 and CLκ domains, and also sequence stretches originating from CH3 domains in order to retain FcRn binding and thus enable FcRn-mediated drug recycling in vivo. In contrast to most of the described Fc heterodimerization technologies (for reviews, see refs. 40,41), which rely on the replacement of single or multiple amino acids located at the CH3–CH3 interface, the herein presented Fc heterodimerization technology is based on the exchange of larger amino acid sequence stretches obtained from human antibody sequences. This approach hypothetically has a lower probability to create de novo T cell epitopes, although this needs to be verified in further studies.

The degree of heterodimerization, mediated by the newly generated Fc1κ, was demonstrated on the basis of asymmetric scFv-Fc1κ fusion proteins and compared to the knobs-into-holes technology, 35 a state-of-the-art heterodimerization platform. In the composition carrying the scFv moiety on the N-terminus of the CH31-containing Fc chain, heterodimer formation was similar compared to knobs-into-holes with the scFv fused to the “holes” Fc chain or even superior compared to knobs-into-holes with the scFv fused to the N-terminus of the “knobs” Fc chain. Notably, in a recently published study on improved versions of the BEAT-Fc part, both asymmetric dAb Fc fusion proteins based on the classic BEAT technology and those based on the SEEDbody technology revealed the residual formation of homodimers. 42 This observation further supports the exceptional heterodimerization quality of the newly generated Fc1κ.

The isolated Fc chain, comprising the CH31 domain, was expressed poorly, even in the presence of the Fc chain containing scFv and CH3κ moieties. This inefficient protein expression might be due to an altered interaction with the luminal endoplasmic reticulum chaperone BiP, which is involved in the processing of the CH1 domain, ensuring that IgG heavy chains are only secreted in combination with the light chain. 43 Similarly, also the AgSb chain of the SEEDbody technology could not be expressed individually; however, this chain does not contain elements of the CH1 domain and was expressed successfully in combination with all other relevant chains of the analyzed Fc-IL-2 fusion protein. 44 Productivity of the Fc1κ chains, and especially of the CH31 comprising chain, will be addressed in detail in the future, such as with respect to cysteine residues, which were demonstrated to control the interaction with the BiP protein and thereby the secretion of IgG heavy chains. 45

The Fv-Fc1κ fusion protein Atrosimab in the composition VL-CH2-CH31/VH-CH2-CH3κ revealed correct heterodimeric assembly without any detectable aggregation or free monomeric polypeptide chains after one-step purification by protein A affinity chromatography, indicating good processability with respect to further drug development. Additional purification steps, such as cation exchange chromatography, recently reported to be required to remove unwanted homodimers during the purification process of a bispecific molecule based on a charge-directed Fc heterodimerization technology, 46 might be included to remove residual side products (for review, see Refs. 47,48).

Atrosimab revealed an aggregation temperature of 64°C, which is similar to that of intact IgG molecules when determined by dynamic light scattering. 49,50 However, there might still be space for optimization, especially considering newly generated interfaces between individual constant Ig domains, which have been demonstrated to represent an efficient target for stability engineering in the case of an scFv to Fab conversion 51 or the fusion of VH or VL to the N-terminus of a CH3 domain. 52

Atrosimab bound to TNFR1 with high affinity (Kd, 2.7 nM) and strongly inhibited TNF-induced receptor activation with IC50 values ranging from 16 nM to 55 nM in different in vitro assays. Compared to the parental Fab 13.7 molecule, TNFR1 binding and inhibition of activation was slightly reduced. Since correct assembly of the heterodimeric protein was confirmed by mass spectrometry analysis, the reduced binding and bioactivity might rather be attributed to alterations in the VH and VL pairing after fusion to the CH2 domain. Similar effects are described in the literature, such as for the conversion of an anti-scorpion toxin scFv into a Fab molecule 53 or an anti-transforming growth factor β1 scFv into an IgG4,54 and has been studied intensively in a comprehensive computational and experimental approach of variable domain exchange in the case of an anti-CD20 scFv. 55

In order to disable the induction of antibody-mediated effector functions via the complement system or natural killer cells, Atrosimab was equipped with a “silent” Fc 56 unable to interact with FcyRI, FcyIIb and FcyIII, as well as with the

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**Table 2. Pharmacokinetic analysis of Atrosimab.**

| Parameter | Value |
|-----------|-------|
| t1/2α, initial half-life | 2.2 ± 1.2 |
| t1/2β, terminal half-life | 41.7 ± 18.1 |
| C0 (µg/ml) | 324.7 ± 53.5 |
| AUC 0-t (µg/ml × h) | 5,856.0 ± 1,369.9 |
| Vss (µg/µg/ml) | 3.4 ± 1.3 |
| CL (µg/(µg/ml)/h) | 0.29 ± 0.21 |

**Figure 9. Pharmacokinetic analysis of Atrosimab.** Four hundred micrograms of Atrosimab were injected into C57BL/6J knock-in mice, carrying the gene of the human TNFR1 extracellular domain connected to the mouse transmembrane and intracellular domains instead of the wild-type mouse gene. Remaining intact protein in the serum was determined by ELISA for binding to TNFR1 at the indicated time points. Shown are the mean ± SD of five mice.
complement protein C1q. Furthermore, this lack of binding to effector molecules of the immune system should also prevent the activation of TNFR1 due to secondary crosslinking of Atrosimab bound to cells expressing FcγRs, as described to be indispensable for antibody-mediated activation of different members of the TNFR superfamily, e.g., CD134. In addition, secondary crosslinking could be mediated by natural or induced ADAs, which have been reported to be a general phenomenon in patients treated with biopharmaceuticals. More particularly, ADAs were observed in the case of patients treated with either infliximab or adalimumab at rates of 50% or 31%, respectively. The detrimental effects of ADAs on anti-TNFR1 agents became obvious in a clinical Phase 1 study concerning a VH dAb (GSK1995057). Moderate symptoms of a cytokine release syndrome (CRS) at doses of 2 to 10 µg/kg were observed due to pre-existing ADAs as determined to be present in 50% of drug naïve patients in subsequent analyses. Atrosimab demonstrated a lack of activation of TNFR1, even in the presence of three different goat anti-human IgG sera. Together with the lack of binding to FcγRs and the complement protein C1q, these data suggest an absence or reduced propensity to cause systemic activation of TNFR1, even in patients with ADAs in their circulation.

Furthermore, Atrosimab revealed a terminal half-life of 41.8 h in C57BL/6J-huTNFRSF1A<sub>tm1UEG</sub>/izj mice after injection of 20 mg/kg body weight, representing an almost 40-fold extension compared to the terminal half-life of Fab 13.7 (1.4 h). Surprisingly, even when compared to ATROSAB (32.1 h), Atrosimab had a 1.3-fold elongated terminal half-life. However, the data concerning Fab 13.7 and ATROSAB were collected after injection of a lower dose (1.25 mg/kg), which might have an impact on the PK characteristics, such as on target-mediated clearance as shown for other antibodies directed against broadly expressed targets. This effect depends on the applied dose, and is detectable only at doses below a drug-specific threshold of target saturation. Conclusively, additional experiments using identical doses of the different agents must be performed to identify the exact PK properties of Atrosimab in comparison to Fab 13.7 and ATROSAB.

In summary, Atrosimab revealed strong binding to TNFR1 and sustained inhibition of TNF-mediated TNFR1 activation in combination with a complete lack of agonistic activity, even in the presence of cross-linking antibodies. Atrosimab furthermore showed a clearly improved in vivo serum circulation time. Regarding the therapeutic application of Atrosimab, it is important to mention that blockade of TNFR1 also inhibits the activity of Lymphotixin-α, which contributes to the inflammatory condition in experimental allergic encephalomylitis in mice and was further shown to be elevated in the synovium of RA patients. Lymphotixin α as a relevant therapeutic target is apparent from a case report on the successful treatment of an infliximab-resistant RA patient with etanercept, the only approved anti-TNF drug that also inhibits Lymphotixin-α activity. Furthermore, long-lasting blockade of complete TNF activity by treatment with classic TNF inhibitors are accompanied by various side effects like susceptibility to infections or, more rarely, the exacerbation of inflammatory symptoms and the development of malignancies. In contrast, selective inhibition of TNFR1, leaving TNFR2 signaling unaffected, represents a more specific intervention in TNF’s pathophysiological actions, both in diseases where anti TNFs show therapeutic activity and those where anti-TNF blockade failed. Moreover, recent publications revealed the involvement of TNFR1 in mouse models concerning tumor lymphangiogenesis and metastasis and in gastric tumorogenesis, which further underlines the potential of Atrosimab as a candidate for treatment not only of inflammatory diseases but also of certain oncological indications.

### Materials and methods

#### Materials

ATROSAB and human TNFR1-Fc were provided by Baliopharm (Basel, Switzerland). The final Atrosimab batch was produced by Catalent (Catalent Pharma Solutions, Somerset, Ewing, NJ, US) in CHO cells after lentiviral transduction and purified by protein A affinity chromatography. Anti-His-horseradish peroxidase (HRP) (HIS-6 His-Probe-HRP, sc-8036) was acquired from Santa Cruz Biotecnonogy (Santa Cruz, CA, USA), anti-human IgG A (Goat, polyclonal, 2010–01) from SouthernBiotec, anti-human IgG B (Goat, polyclonal, MBS571163) and anti-human IgG B (Goat, polyclonal, MBS571678) from MyBioSource, (San Diego, CA, USA), anti-human IgG (Fab specific, A 0293) and anti-human IgG (Fc specific, A 0170) from Sigma-Aldrich (Taufkirchen, Germany).

#### Protein production

DNA encoding the interspersed constant Ig domains connected to the C-terminus of an IgG CH2 domain were ordered from GeneArt® (Regensburg, Germany) and cloned into the expression vector pSecTagAL1 (modified from pSecTag-FcHis), which already contained the variable domains of the heavy or light chain of Fab 13.7 using the restriction enzymes Kpn1 and EcoRI. All Atrosimab variants and the Fab 13.7 protein were produced in HEK293E cells after transient transfection of a mixture of two vectors, encoding for the different chains of the dimeric molecules using polyethylenimine (linear, 25 kDa, Sigma-Aldrich, Taufkirchen, Germany). Proteins were purified by protein A affinity chromatography according to the manufacturers’ protocol (TOYOPEARL®, AF-rProtein A-650F, 22805, Tosoh, Taufkirchen, Germany). In the case of the Atrosimab batch produced by Catalent (Catalent Pharma Solutions, Somerset, Ewing, NJ, US), a preparative gel-filtration step was performed (Äkta purifier, Superdex 200 10/300 GL column, flow rate of 0.5 ml/min, phosphate-buffered saline (PBS) as liquid phase).

#### Protein characterization

Purified protein samples were analyzed by SDS-PAGE using 4 µg of each sample under reducing and non-reducing conditions. Proteins were stained using Coomassie-Brilliant Blue G-250 and acrylamide gels were de-stained with water. Correct assembly under native conditions was visualized by SEC using a Waters 2695 HPLC and a Phenomenex Yarra SEC-2000 column (300 x 7.8 mm) or a TSKgel SuperSW mAb HR column.
(flow rate of 0.5 ml/min, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 6.7 as mobile phase). Standard proteins (MW, r₅₀): Thyroglobulin (669 kDa, 8.50 nm), Apoferritin (443 kDa, 6.10 nm), beta Amylase (200 kDa, 5.4 nm), bovine serum albumin (67 kDa, 3.55 nm), Carbonic anhydrase (29 kDa, 2.35). Mass spectrometry analysis was performed by Xin Chen at Catalent Pharma Solutions (New Jersey, US) after deglycosylation using PNGase F (1 unit/µg protein) for 18 h at 37°C. Separation was performed on an ACQUITY UPLC® H-Class System (Waters; Milford, MA). Measurements were implemented on a Xevo G2-XS system with an electrospray ionization (ESI) source (Waters; Milford, MA). The system was controlled by UNIFI 1.8 (Waters; Milford, MA). The prepared samples were injected into a Waters BEH C4 column (2.1 x 100 mm, 1.7 µm) at an injection volume of 5 µL. Mobile Phase A was water with 0.1% formic acid (FA) and Mobile Phase B was acetonitrile with 0.1% FA. A linear gradient was used.

**Thermal stability**

Temperature-dependent aggregation of Atrosimab was analyzed using the ZetaSizer Nano ZS (Malvern, Herrenberg, Germany). Increasing temperatures were applied stepwise from 35°C to 80°C with intervals of 1°C and equilibration times of 2 min prior to each measurement. One hundred micrograms of protein were diluted in 1 ml PBS and the melting/aggregation temperature (Tm) was determined by visual interpretation of the increasing signal of the particle size (kcps).

**Plasma stability**

Atrosimab samples were diluted to 100 nM in human plasma, incubated at 37°C for 1, 3 and 7 days and subsequently analyzed by ELISA for binding to human TNFR1 (as described below) after serial dilution in 2% skim milk in PBS (2% MPBS) by steps of 1 to 3.16 (square root of 10). A protein sample, stored at 4°C and a protein sample, frozen immediately after dilution in human plasma, were used as controls.

**Enzyme-linked immunosorbent assay**

Indicated proteins were diluted to 1 µg/ml in PBS, transferred to a 96-well microtiter plate and incubated overnight at 4°C. Skim milk in PBS (2% MPBS) was used to block residual binding sites (200 µl/well at room temperature (RT)). All samples were diluted in 2% MPBS to the indicated maximal concentrations and diluted in steps of 1 to 3.16 (square root of 10). Each sample was transferred to the microtiter plates and incubated at RT for another hour. HRP-labeled detection antibodies were diluted in 2% MPBS as recommended by the manufacturer and applied to the plates for 1 h at RT. Binding of the analyzed proteins was detected using 100 µl substrate solution (1 mg/ml 3,3′,5,5′-Tetramethylbenzidine[TMB], 0.006% H₂O₂ in 100 mM Na-acetate buffer, pH 6 at RT) and the detection reaction was stopped using 50 µl 1 M H₂SO₄. Absorption was determined at a wavelength of 450 nm. In each step, a working volume of 100 µl was used and between each step, the plates were washed twice with PBS containing 0.005% Tween20 (PBST) and twice with PBS.

**Quartz crystal microbalance**

Real-time binding kinetics were analyzed using the A-100 C-Fast or Cell-200 C-Fast biosensors (Attana, Stockholm, Sweden). A human TNFR1-Fc fusion protein was covalently immobilized to the Attana LNB Carboxyl Sensor Chip (3623–3103, Attana, Stockholm, Sweden) at the indicated ligand density, using the Amine Coupling Kit (3501–3001, Attana, Stockholm, Sweden) as recommended by the manufacturer. Binding of analytes was performed at 37°C, using PBST (0.1% Tween-20, pH 7.4) as running buffer at a flow rate of 25 µl/min. Reference injection of running buffer was performed after every other measurement and subtracted from the binding curves during data evaluation using the Attaché Office Evaluation software (Attana, Stockholm, Sweden) and TraceDrawe (Ridgeview instruments, Vange, Sweden). Sensor chip regeneration was accomplished by injecting 20 mM glycine, pH 2.0 twice for 12 s.

**Interleukin release assay**

HeLa (IL-6) or HT1080 (IL-8) cells were adjusted to a concentration of 2 x 10⁶ cells/ml and 100 µl were distributed in 96-well plates and incubated at 37°C, 5% CO₂ overnight. The used medium (RPMI 1640 + 5% FCS) was discarded subsequently to remove present IL-6 or IL-8, and the protein samples were diluted in culture medium and applied to the cells. In the case of inhibition experiments, antibody samples were added to the cells immediately following to the addition of the stimulant (TNF). After 16–20 h of incubation at 37°C, 5% CO₂, the supernatants were harvested subsequent to 5 min of centrifugation at 500 x g, diluted in culture media (stimulation experiments 1:2, competition 1:25, 33 nM TNF control 1:75) and analyzed for IL-6/IL-8 concentration by ELISA as recommended by the manufacturer (IL-6, 31670069, IL-8, 31670089, ImmunoTools, Friesoythe, Germany).

**Cytotoxicity assay**

Kym-1 cells were incubated overnight at 37°C and 5% CO₂ in 96-well microtiter plates (1 x 10⁴ per well) and subsequently treated with serial dilutions of the protein samples in culture medium (5% RPMI 1640 + 10% FCS) alone or in combination with a constant concentration of TNF (0.01 nM) for another 24 h at 37°C and 5% CO₂. The supernatants were discarded and 50 µl crystal violet solution (0.5% crystal violet, 20% methanol in H₂O) was added to each well and incubated for 20 min at RT. Plates were washed 20 times with ddH₂O, dried at RT, and residual crystal violet was dissolved in 50 µl methanol per well. The absorption at 595 nm was measured after shaking the plates for 10 min at RT.

**Pharmacokinetics**

Animal care and experiments were performed in accordance with federal guidelines and have been approved by university
and state authorities. PK properties of Atrosimab were determined after injection of 400 μg protein into the tail vein of transgenic C57BL/6 mice expressing a chimeric TNFR1, composed of the extracellular domain of human TNFR1 and the murine intracellular region from the locus of the particular mouse gene (C57BL/6J-huTNFRSF1AeecdmgE/izi). Blood samples were collected at the indicated time points from the tail and incubated on ice for 20 min. Serum was separated by centrifugation (13,000 x g, 4°C, 10 min) and frozen to −20°C prior to analysis. Binding of remaining protein in the serum samples to human TNFR1 was analyzed by ELISA and the resulting data were analyzed using the PKSolver Excel add-in.

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Disclosure of Potential Conflicts of Interest

F.R., O.S., A.H., K.P., and R.E.K. are named inventors on patent applications covering Fc heterodimerization modules and monovalent inhibitors of TNFR1 interaction.

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Abbreviations

ADA anti-drug antibodies
ATROSAB antagonistic TNF receptor one specific antibody
AUC area under the curve
CH1 first constant domain of the IgG1 heavy chain
CH3 third constant domain of the IgG1 heavy chain
CH3κ CH3 kappa
CHO Chinese hamster ovary
CLA collagen-induced arthritis
CLk constant domain of the kappa light chain
CRS cytokine release syndrome
dAb domain antibody
EAE experimental autoimmune encephalomyelitis
ELISA enzyme-linked immunosorbent assay
ESI electrospray ionization
FA formic acid
Fab antigen-binding fragment
Fcy1κ Fc-one kappa
Fcrn neonatal Fc receptor
Fv fragment variable
HRP horseradish peroxidase
Ig immunoglobulin
IL-6 interleukin-6
IL-8 interleukin-8
LPS lipopolysaccharides
LT Lymphotoxin
M Marker
NR non-reducing conditions
PBS phosphate-buffered saline
PBST PBS containing Tween20
PEG polyethylene glycol
PK pharmacokinetic
QCM quartz crystal microbalance
R reducing conditions

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