Charge-mediated influence of the antibody variable domain on FcRn-dependent pharmacokinetics

Angela Schoch*, Hubert Kettenbergerb, Olaf Mundiglc, Gerhard Winterd, Julia Engertd, Julia Heinricha, and Thomas Emrich*b,c

aLarge Molecule Bioanalytical Research and Development, Pharmaceutical Sciences, bLarge Molecule Research Biochemical and Analytical Research, Large Molecule Research, cLarge Molecule Research Discovery, Large Molecule Research, Roche Pharmaceutical Research and Early Development, Roche Innovation Center Penzberg, 82377 Penzberg, Germany; and dInstitute of Pharmaceutical Technology and Biopharmaceutics, Ludwig Maximilians University, 81377 Munich, Germany

Here, we investigated the influence of the variable fragment (Fv) of IgG antibodies on the binding to the neonatal Fc receptor (FcRn) as well as on FcRn-dependent pharmacokinetics (PK). FcRn plays a key role in IgG homeostasis, and specific manipulation in the crystallizable fragment (Fc) is known to affect FcRn-dependent PK. Although the influence of the antigen-binding fragment (Fab) on FcRn interactions has been reported, the underlying mechanism is hitherto only poorly understood. Therefore, we analyzed the two IgG1 antibodies, briakinumab and ustekinumab, that have similar Fc parts but different terminal half-lives in human and systematically engineered variants of them with cross-over exchanges and varied charge distribution. Using FcRn affinity chromatography, molecular dynamics simulation, and in vivo PK studies in human FcRn transgenic mice, we provide evidence that the charge distribution on the Fv domain is involved in excessive FcRn binding. This excessive binding prevents efficient FcRn–IgG dissociation at physiological pH, thereby reducing FcRn-dependent terminal half-lives. Furthermore, we observed a linear correlation between FcRn column retention times of the antibody variants and the terminal half-lives in vivo. Taken together, our study contributes to a better understanding of the FcRn–IgG interaction, and it could also provide profound potential in FcRn-dependent antibody engineering of the variable Fab region.

Human IgGs contain two antigen-binding (Fab) regions that convey specificity for the target antigen and a constant (Fc) region that is responsible for interactions with Fc receptors (1, 2). Human IgGs of subclasses 1, 2, and 4 have an average serum half-life of 21 days (d), which is longer than that of any other known serum protein (3). This long half-life is predominantly mediated by the interaction with the neonatal Fc receptor (FcRn) (4, 5) and is one of the reasons why IgGs or Fc-containing fusion proteins are widely used as therapeutic proteins.

FcRn is a membrane-associated receptor involved in both IgG and albumin homeostasis, in maternal IgG transport across the placenta, and in antigen–IgG immune complex phagocytosis (6–8). Human FcRn is a heterodimer consisting of the glycosylated α-FcRn and a β2 microglobulin (β2m) subunit (9). FcRn binds to a site in the C1r2-C1s3 region of the Fc region (10–13), and two FcRn molecules can bind to the Fc region simultaneously (14, 15). The affinity between FcRn and Fc is strongly pH dependent, showing nanomolar affinity at endosomal pH of 5–6 and negligible binding at a physiological pH of 7.4 (16, 17). The underlying mechanism conveying long half-life to IgGs can be explained by three fundamental steps. First, IgGs are subject to unspecific pinocytosis by various cell types (18, 19). Second, IgGs encounter and bind FcRn in the acidic endosome at a pH of 5–6, thereby protecting IgGs from lysosomal degradation (10, 17, 20). Finally, IgGs are released in the extracellular space at physiological pH of 7.4 (4). This strict, pH-dependent bind-and-release mechanism is critical for IgG recycling, and any deviation of the binding characteristics at different pHs may strongly influence circulation half-lives of IgGs (21).

In addition to the specific interaction of the Fc region with FcRn, the Fab regions have also been suggested to contribute to FcRn binding (22–24). For example, Fab-mediated residual binding at near physiological pH was correlated with the pharmacokinetic properties of a set of therapeutic antibodies, indicating that IgGs with excessive binding to FcRn at pH 7.3 suffer from reduced terminal half-lives (23). Recently, Schlothauer et al. (24) have described a novel pH gradient FcRn affinity chromatography method that closely mimics physiological conditions for the dissociation between FcRn and IgGs. Furthermore, they showed that IgGs with identical Fc regions differ in their dissociation from FcRn, thereby indicating the influence of the Fab region on FcRn binding. However, the underlying mechanism how the distal Fab region influences FcRn binding is hitherto only poorly understood.

To systematically investigate the influencing factors of the Fab region to FcRn-mediated IgG homeostasis, we used the antibody pair briakinumab (Ozespa) and ustekinumab (Stelara) as a model system. Both briakinumab and ustekinumab are fully human monoclonal IgG1 antibodies that specifically bind to the human p40-subunit of interleukin 12 and interleukin 23 (25) and not to the corresponding mouse interleukin 12 and interleukin 23. Both antibodies have nearly identical constant IgG1 domains, with minor differences in several allotype-specific amino acids in this region (Fig. S1). However, these different amino acids are outside of the cognate FcRn-binding regions and therefore are considered to play no significant role in FcRn-dependent FcRn | pharmacokinetics | antibody | charge | engineering

Significance

Therapeutic antibodies of the immunoglobulin G (IgG) isotype show a pharmacokinetic (PK) profile that is strongly mediated by the interaction with the neonatal Fc receptor (FcRn). Therefore, modulating the FcRn–IgG interaction allows altering PK characteristics of therapeutic antibodies. So far, engineering the crystallizable fragment (Fc) is known to affect PK, and, although the influence of the antigen binding fragment (Fab) on FcRn interactions has been reported, the underlying mechanism remains unknown. Here, we demonstrate that the charge distribution in the distal variable fragment (Fv) of IgGs is involved in excessive binding to the FcRn, thereby reducing FcRn-dependent terminal half-lives in vivo. These findings contribute to a better understanding of the FcRn–IgG interaction.

Author contributions: A.S., H.K., O.M., G.W., J.H., and T.E. designed research; A.S., H.K., O.M., J.E., and T.E. performed research; A.S. and T.E. analyzed data; and A.S., H.K., O.M., J.H., and T.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Frequently available online through the PNAS open access option.

*To whom correspondence should be addressed. Email: thomas.emrich@roche.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1408766112/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1408766112
PNAS | May 12, 2015 | vol. 112 | no. 19 | 5997–6002

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pharmacokinetics (PK) (10). Briakinumab is an IgG1 antibody with variable heavy and light chain domains of the V_{H}3 and V_{L}1 germ-line families. Ustekinumab is an IgG1 antibody with variable heavy and light chain domains of the V_{H}5 and V_{L}1D germ-line families. Interestingly, ustekinumab has a median terminal half-life of 22 d (26) whereas briakinumab has a terminal half-life of only 8–9 d (25, 27, 28). Therefore, we used briakinumab and ustekinumab as our model system to elucidate the role of the Fab region on FcRn-dependent PK.

Here, we report that the charge distribution in the variable fragment (Fv) domain is involved in excessive FcRn binding, thereby reducing FcRn-dependent terminal half-lives. To elucidate which structural element of the Fab region and how the Fab region influences FcRn binding, we engineered nine variants of briakinumab and ustekinumab. Furthermore, we analyzed all 11 antibodies using a combination of in vitro FcRn affinity chromatography, molecular dynamics (MD) simulation, subcellular trafficking analysis by confocal microscopy, and in vivo PK studies in human FcRn transgenic mice.

Results

Charge Distribution and pH-Dependent Net Charge. Using the published crystal structure of ustekinumab (29) and a homology model of briakinumab, we observed that briakinumab exhibits an unusual, nonuniform charge distribution at physiological pH of 7.4. Briakinumab shows a large positively charged region on the Fv domain (Fig. 1D) that is absent in ustekinumab (Fig. 1B). Furthermore FcRn was found to possess an extended negatively charged region (Fig. 1C) that is, however, not involved in cognate Fc binding. Briakinumab and ustekinumab have a similar isoelectric point of 9.6 and 9.3, respectively, whereas the net charge of briakinumab is slightly more positive over the entire pH range (Fig. 1D) than the net charge of ustekinumab. Therefore, we hypothesized that the FcRn–Fv interaction could be charge-mediated.

pH-Dependent FcRn–IgG Interaction. To investigate whether the reduced terminal half-life of briakinumab is caused by an altered binding to FcRn involving the positively charged (briakinumab) and negatively (FcRn) charged regions, we constructed nine variants of briakinumab and ustekinumab (Table 1). In particular, we systematically modified the variable regions and tested their influence on FcRn binding at pH 6.0 and on FcRn dissociation using surface plasmon resonance (SPR) and FcRn affinity chromatography (Table 2), respectively.

The mAb 5, an antibody at pH 6.0 fell in a narrow range for all 11 antibodies (Table 2). The equilibrium dissociation constant ($K_D$) was calculated relative to ustekinumab (ustekinumab = 1.0). Briakinumab had a relative $K_D$ of 0.2, and the nine variants ranged between briakinumab and ustekinumab. Due to this rather small $K_D$ difference, we concluded that different terminal half-lives are not caused by different FcRn binding at pH 6.0.

To mimic the FcRn–IgG dissociation at physiological pH, we analyzed the elution profiles of the 11 antibodies using an FcRn-affinity column with pH gradient elution (Fig. 2). Ustekinumab and mAb 1, which bears the Fv domain of ustekinumab on the constant parts of briakinumab, showed indistinguishable retention times at around 84 min, indicating that the Fv domain influences the interaction with the FcRn. Briakinumab, on the other hand, eluted at a retention time of 94 min and therefore showed a clear difference in retention time compared with ustekinumab. The indistinguishable retention times of the immunoglobulin G-degrading enzyme of Streptococcus pyogenes (IdeS)-cleaved Fc regions of briakinumab (85.7 min) and ustekinumab (85.2 min) supported the negligible role of the Fc region in this setting. The mAb 4 containing ustekinumab light chains (LCS) and briakinumab heavy chains (HCS) eluted close to ustekinumab, revealing the impact of the LC on FcRn binding in this model system. To study the influence of the complementarity-determining regions (CDRs) on FcRn dissociation, we constructed variants mAb 5 and mAb 6 that bear ustekinumab CDRs on the briakinumab framework and vice versa. Grafting ustekinumab CDRs on briakinumab (mAb 5) shifted the retention time of mAb 5 close to that of ustekinumab. However, the opposite was not observed to the same extent. Briakinumab CDRs on ustekinumab (mAb 6) described an elution profile that was still close to ustekinumab. A strong retention time shift from briakinumab in the direction of ustekinumab was observed for mAb 9, which is a briakinumab variant in which three positively charged residues in the light chain CDRs were mutated to alanines. Three and five positively charged residues in the heavy chain of briakinumab were mutated in mAb 7 and mAb 8, respectively. Here, the retention time shift relative to briakinumab was not as strong as for mAb 9. The mAb 3, comprising the HCs of ustekinumab and the LCS of briakinumab, as well as mAb 2 containing the Fv domain of briakinumab on the ustekinumab constant domains, both eluted close to briakinumab. Taken together, we showed that the Fv domain influences FcRn dissociation and not FcRn binding at pH 6.0.

To study in more detail the nature of the FcRn–Fv interaction, we tried to correlate the FcRn column retention times with calculated isoelectric points and net charges of the antibodies. We did not observe a correlation between the FcRn column retention times and the isoelectric points or the net charges of the Fv domains at lysosomal pH 6.0 or physiological pH 7.4 (Table 2). However, we observed that the FcRn column retention times increased with the extent of positively charged regions on the Fv structures, especially around the light chain variable domains (Fig. 2). We next measured FcRn column retention times in the presence of increasing salt concentrations because charge-mediated interactions are known to be weakened under high ionic strength conditions. We observed that the FcRn column retention time of briakinumab was shortened in the presence of salt in a manner proportional to the inverse square root of the ionic strength, as suggested by the Debye–Hückel law of charge screening (30). Conversely, the retention time of ustekinumab remained essentially unaffected (Fig. S2). Therefore, we demonstrated that a significant part of the excessive FcRn–briakinumab interaction is charge-mediated.

Correlation Between In Vivo PK Parameters and FcRn Column Elution pHs. To assess whether the effect of mutated charged residues in the variable domains of briakinumab on FcRn binding translates
within modulated in vivo PK properties, we conducted PK studies in human FcRn transgenic mice. Briakinumab and ustekinumab, together with two variants of briakinumab (mAb 8 and mAb 9), which had FcRn column retention times between briakinumab and ustekinumab, were tested. Mean serum concentration-time profiles for the antibodies are shown in Fig. 3d. As expected, briakinumab and ustekinumab significantly differ in their PK behavior, showing terminal half-lives of 48 h and 137 h, respectively (Fig. 3b). Briakinumab also showed a faster decrease in the α-phase that might be caused by different distribution processes. In our study, the variants mAb 8 and mAb 9, which have smaller positively charged regions in the Fv domain compared with briakinumab, had terminal half-lives of 78 h and 109 h, respectively. A statistical significance could be detected between the terminal half-lives of briakinumab and ustekinumab and of briakinumab and mAb 9, as well as of ustekinumab and mAb 8. Ustekinumab, mAb 9, mAb 8, and briakinumab eluted at 84.3, 86.2, 90.1, and 93.7 min, corresponding to an elution pH of 7.4, 7.5, 7.7, and 7.9, respectively. Importantly, the terminal half-lives of the four IgGs were linearly correlated with the in vitro FcRn column elution pHs (Fig. 3b).

Subcellular Trafficking Analysis. To analyze the influence of the differently charged Fv regions of briakinumab and ustekinumab at the cellular level, we incubated human umbilical vein endothelial cells (HUVECs) with the directly fluorescently labeled IgGs to allow fluid phase pinocytosis under physiological conditions. Within 40 min of incubation (20-min loading period followed by a 20-min chase period), both briakinumab and ustekinumab could be found in distinct subdomains of FcRn-positive sorting endosomes (17, 31), indicating that the two IgGs are differentially sorted already at this level (Fig. 4).

Molecular Dynamics Simulation of the FcRn–IgG Models. To further support the hypothesis of a charge-mediated Fab–FcRn interaction, we studied the dynamics of the FcRn–IgG complexes by MD simulation. We used the recently published structure of human FcRn in complex with a human Fc and serum albumin (PDB ID code 4N0U). We superimposed this complex (omitting serum albumin) with the crystal structure of a complete IgG1 (PDB ID code 1H2Z) and with the X-ray structure of the Fab fragment of ustekinumab (PDB ID code 3HMX). Because no experimental structure of briakinumab was available, we used a homology model instead (Materials and Methods). These IgG–FcRn complex models contain two copies of FcRn (α-FcRn with β2m) on one complete IgG molecule (Figs. 1C and 5A). The distance between the FcRn and the Fv domains is 40 Å in the starting structure and thus exceeds the Debye length of ∼1 Å under physiological conditions (30). Consequently, the Fv domain must approach the FcRn to establish an attractive interaction. To study whether conformational flexibility of the IgG hinge region supports such domain movements, we calculated the molecular dynamics of the briakinumab and ustekinumab FcRn–IgG complexes over a period of 100 ns in explicit water. During the course of the simulation, one of the two Fab arms of briakinumab approached the tip of FcRn and persisted in this conformation for the rest of the simulation time (Fig. 5A and D). This finding suggests that the conformational flexibility of the Fab arms allows them to readily assume conformations that can stably interact with FcRn. The region on FcRn found to interact with the Fv domain has not been described as being involved in IgG binding before. In this MD simulation, the Fab arms of ustekinumab did not approach FcRn but randomly moved in conformations without particular preferences (Fig. 5A, B, and D). In summary, MD simulation indicates that the flexibility of Fab arms in the FcRn–IgG complexes structurally allows a direct, stabilizing interaction of the Fv domain with the tip of FcRn.

Discussion

Specific manipulation of the Fc region is known to affect PK characteristics by altering the interaction between the Fc region and FcRn, especially at pH 6.0, and has been used to design therapeutic antibodies with specific PK properties (32, 33). Although the influence of the Fab region on FcRn interactions has recently been discussed, when antibodies of the same WT human Fc sequences but different Fab regions showed differences in FcRn binding and altered PK, the underlying mechanism of this interaction remained unclear (22, 23). In this study, we provide

### Table 1. Systematically engineered variants of briakinumab and ustekinumab

| Name                | Description                                                                 |
|---------------------|----------------------------------------------------------------------------|
| Briakinumab         | Briakinumab                                                                |
| Ustekinumab         | Ustekinumab                                                                |
| mAb 1               | Ustekinumab Fv + briakinumab constant domains                              |
| mAb 2               | Briakinumab Fv + ustekinumab constant domains                              |
| mAb 3               | Ustekinumab HC + briakinumab LC                                            |
| mAb 4               | Briakinumab HC + ustekinumab LC                                            |
| mAb 5               | Ustekinumab CDRs on briakinumab                                           |
| mAb 6               | Briakinumab CDRs on ustekinumab                                           |
| mAb 7               | Briakinumab R19HCA, K64HCA, R83HCA*                                       |
| mAb 8               | Briakinumab R16HCA, R19HCA, K57HCA, K64HCA, R83HCA*                       |
| mAb 9               | Briakinumab R27LCA, R55LCA, R94LCA*                                       |

Structural parts like Fv, LC, and CDRs were exchanged between briakinumab and ustekinumab: mAb 1–6. Three and five basic amino acids in the HC of briakinumab were exchanged into alanines for mAb 7 and mAb 8, respectively. MAb 9 is briakinumab with three basic amino acids in the LC CDRs exchanged into alanines. Asterisks (*) mark exchanged amino acid residues according to the EU numbering of Kabat (45).

### Table 2. FcRn affinities and calculated net charge of all tested antibodies

| Name            | Ret. time (min) | Rel. Kd | pi (IgG) | q(Vs) pH 6 | q(Vh) pH 7.4 | q(Vs) pH 6 | q(Vh) pH 7.4 | q(Fv) pH 6.0 | q(Fv) pH 7.4 |
|-----------------|----------------|---------|----------|------------|--------------|------------|--------------|--------------|--------------|
| Ustekinumab     | 84.3           | 1       | 9.3      | 2.1        | 1.9          | 3.1        | 2.9          | 5.2          | 4.9          |
| mAb 1           | 84.3           | 1.0 ± 0.22 | 9.5    | 2.1        | 1.9          | 3.1        | 2.9          | 5.2          | 4.9          |
| mAb 2           | 84.5           | 0.5 ± 0.08 | 9.6    | 2.1        | 1.9          | 4.1        | 3.9          | 6.1          | 5.9          |
| mAb 4           | 85.1           | 0.9 ± 0.16 | 9.9    | 2.1        | 1.9          | 4.1        | 3.9          | 6.1          | 5.9          |
| mAb 5           | 86.2           | 0.4 ± 0.17 | 9.0    | 3.9        | 3.0          | 5.4        | 3.3          | 9.2          | 6.3          |
| mAb 6           | 86.2           | 0.4 ± 0.04 | 9.1    | 0.8        | 0.0          | 6.4        | 4.3          | 7.2          | 4.3          |
| mAb 7           | 90.1           | 0.4 ± 0.07 | 8.8    | 3.8        | 3.0          | 6.4        | 4.3          | 7.2          | 4.3          |
| mAb 8           | 90.4           | 0.2 ± 0.03 | 9.2    | 3.8        | 3.0          | 6.4        | 4.3          | 7.2          | 4.3          |
| mAb 9           | 92.4           | 0.2 ± 0.06 | 9.3    | 3.8        | 3.0          | 6.4        | 4.3          | 7.2          | 4.3          |
| mAb 2           | 93.0           | 0.3 ± 0.19 | 9.3    | 3.8        | 3.0          | 6.4        | 4.3          | 7.2          | 4.3          |
| Briakinumab     | 93.7           | 0.7 ± 0.07 | 9.6    | 3.8        | 3.0          | 6.4        | 4.3          | 7.2          | 4.3          |

Antibodies are sorted according to the FcRn column retention times (Ret. time). The equilibrium dissociation constant Kd was calculated as steady-state affinity and normalized to the Kd of ustekinumab. Relative (Rel.) Kd values (ustekinumab = 1) are presented as the mean (n = 3) ± SD.
previous studies have discussed net charge to be a driving force for altered PKs by affecting the electrostatic interaction between the antibody and negatively charged groups on the surface of endothelial cells (34, 35). For example, Iwasa et al. (36) observed that IgG4 antibodies with lower isoelectric point (pl) values due to engineering in the variable region have a lower rate of fluid-phase pinocytosis and in turn a reduced elimination rate. Furthermore, Boswell et al. (37) proposed the pl differences needed to be at least of one unit to influence PK. In contrast, the pl values of briakinumab, ustekinumab, and mAb 9 were found to be in a relatively narrow range between 9.3 and 9.6 and the pl of mAb 8 was determined to be 8.8. Therefore, we assume the influence on fluid-phase pinocytosis to be minimal. However, briakinumab’s shorter FcRh column retention times under high ionic strength conditions, as well as the higher electrostatic contribution to the FcRn–Fv interaction compared with ustekinumab, indicated that a specific located charge in the light chain CDRs strongly influences FcRn dissociation.

In our in vivo experiments in human FcRn transgenic mice, we focused on the influence of the Fv domain on FcRn recycling; therefore, we examined the terminal half-life, which is exclusively calculated in the elimination phase where FcRn recycling dominates (38). The terminal half-lives of the four antibodies correlated linearly with the in vitro FcRn column elution pH, thereby indicating the potential of FcRn affinity chromatography to predict Fab-mediated PK properties. The correlation between terminal half-lives and the FcRn column elution pH confirmed the importance of the efficient FcRn–IgG dissociation at physiological pH. A similar finding has been observed for Fc-engineered IgG molecules with improved FcRn binding at both endosomal pH 6.0 and at physiological pH 7.4, which did not show prolonged half-life (39). The FcRn–IgG complex is built in the endosomes at pH 6.0; therefore, less binding results in less IgG recycling and faster clearance. During exocytosis, the FcRn–IgG complex is released to the plasma membrane, where the dissociation of the IgG and the FcRn has to take place at a physiological pH of 7.4 within a short period (40). Consequently, dissociation at physiological pH is also important for a prolonged half-life (21, 40).

In conclusion, our experiment supports the hypothesis that a slower dissociation at higher pH could lead to degradation of the antibodies in the lysosome instead of releasing the antibodies back to blood circulation. In addition, cellular internalization and sorting experiments demonstrate a separation of briakinumab and ustekinumab at the level of the
Fig. 4. Differential sorting of briakinumab and ustekinumab in FcRn-positive sorting endosomes. HUVECs were incubated together with 200 μg/mL both briakinumab (labeled with AlexaFluor594) and ustekinumab (labeled with AlexaFluor488) for 20 min at 37 °C, pH 7.3, followed by 20 min chase. At the end of the experiment, cells were fixed and counterstained with a monoclonal antibody directed against FcRn (DVN22) (44) detected by secondary antibodies labeled with AlexaFluor647.

Fig. 5. Molecular dynamics simulation of FcRn-IgG models. (A) Conformation at the start of the simulation. The dashed line indicates the distance between two example amino acids in the Fv region and in the FcRn, which approach each other during the MD simulation as shown in Fig. 1. (B) Conformation at the end of the simulation (t = 100 ns). The box indicates the part of the molecule shown in B. (C) Detailed view of the interaction between FcRn and the Fv domain of briakinumab. CDRs of the HC and the LC are colored in dark and light purple, respectively. (D) Distance between residues 192 (FcRn) and 57 (ustekinumab LC) and 58 (briakinumab LC), respectively, during the course of the simulation. Protein structures were prepared with PyMol (Schrodinger LLC).
Imaging was performed on a Leica SP8 confocal microscope using hybrid detectors (HyD). Imaging conditions were as follows: 100x/1.46 NA oil immersion lens with sequential acquisition for each channel using white light laser excitation (Ex) at 488 nm/emission (Em) 491–559 nm, 594/640–633 nm, and Ex 633 nm/Em 637–721 nm, respectively. Images were processed by blind deconvolution using AutoQuant X3.0.3 to improve resolution.

Pharmacokinetic Studies. B6.Cg-Fcgr1<tnl−/−> Tg(FcGRT)276Dcr mice deficient in mouse FcRn α-chain gene, but hemizygous transgenic for a human FcRn α-chain gene (mFcRn<sup>−</sup>hFcRn<sup>−</sup> line 276), were used for the pharmacokinetic studies (3B). A single dose of antibody was injected i.v. via the lateral tail vein at a dose level of 10 mg/kg. The mice were divided into three groups of six mice each to cover nine serum collection time points in total (at 0.08, 2, 8, 24, 48, 168, 336, 504, and 672 h post-dose). Each mouse was subjected twice to retro-orbital bleeding, performed under light anesthesia with isoflurane (Pharmacia-GmbH), a third blood sample was collected at the time of euthanasia. Blood was collected into serum tubes (Microvette 5000Gel, Sarstedt). After 2 h incubation, samples were centrifuged for 3 min at 9,300 × g to obtain serum. After centrifugation, serum samples were stored frozen at −20 °C until analysis.

Determination of Human Antibody Serum Concentrations. Concentrations of ustekinumab, briakinumab, mAb B, and mAb 9 in murine serum were determined by specific enzyme-linked immunosassays. Biotinylated interleukin 12 and digoxigenin-labeled anti-human-Fc mouse monoclonal antibody (Roche Diagnostics) were used for capturing and detection, respectively. Streptavidin-coated microtiter plates (Roche Diagnostics) were coated with capture protein diluted in assay buffer (Universal buffer for ELISA; Roche Diagnostics) for 1 h. After washing, serum samples were added at various dilutions, followed by another incubation step for 1 h. After repeated washings, bound human antibodies were detected by subsequent incubation with detection antibody, followed by an anti-digoxigenin antibody conjugated to horseradish peroxidase (HRP) (Roche Diagnostics). ABTS (2,2-azino-di-[3-ethylbenzthiazoline sulfonate]; Roche Diagnostics) was used as HRP substrate to form a colored reaction product. Absorbance of the resulting reaction product was read at 405 nm with a reference wavelength at 490 nm using a Tecan sunrise plate reader. All serum samples and positive and negative control samples were analyzed in duplicate and calibrated against a reference standard using a four-parameter fit.

AKNOWLEDGMENTS. We thank Sabine Imhof-Jung and Petra Rüeger for production of all antibodies used in this work and Doris Ziegler-Landesberger for optimizing the in vitro cell culture models used. Furthermore, we thank Tilman Schlothauer for his support and helpful discussions.