Linker Length Matters, Fynomer-Fc Fusion with an Optimized Linker Displaying Picomolar IL-17A Inhibition Potency*

Received for publication, November 12, 2013, and in revised form, March 24, 2014. Published, JBC Papers in Press, April 1, 2014, DOI 10.1074/jbc.M113.534578

Michela Silacci, Nadja Baenziger-Tobler, Wibke Lembke, Wenjuan Zha, Sarah Batey, Julian Bertschinger, and Dragan Grabulovski

From Covagen AG, Wagistrasse 25, CH-8952 Schlieren, Switzerland

**Background:** IL-17A is a proinflammatory cytokine involved in various inflammatory diseases.

**Results:** Potent IL-17A inhibitors were generated by fusing human Fyn SH3 domain derivatives (Fynomers) to the Fc part of an antibody.

**Conclusion:** The linker length between the Fc part and the binding domain influences significantly the potency of Fc fusion proteins.

**Significance:** Fynomers represent a promising class of therapeutic proteins.

Fynomers are small binding proteins derived from the human Fyn SH3 domain. Using phage display technology, Fynomers were generated inhibiting the activity of the proinflammatory cytokine interleukin-17A (IL-17A). One specific Fynomer called 2C1 inhibited human IL-17A *in vitro* with an IC_{50} value of 2.2 nm. Interestingly, when 2C1 was genetically fused to the Fc part of a human antibody via four different amino acid linkers to yield bivalent IL-17A binding proteins (each linker differed in length), the 2C1-Fc fusion protein with the longest linker displayed the most potent inhibitory activity. It blocked homodimeric IL-17A with an IC_{50} value of 21 pm, which corresponds to a hundredfold improved IC_{50} value as compared to the value obtained with monovalent Fynomer 2C1. In contrast, the 2C1-Fc fusion with the shortest linker showed only an ~8-fold improved IC_{50} value of 260 pm. Furthermore, in a mouse model of acute inflammation, we have shown that the most potent 2C1-Fc fusion protein is able to efficiently inhibit IL-17A *in vivo*. With their suitable biophysical properties, Fynomer-Fc fusion proteins represent new drug candidates for the treatment of IL-17A mediated inflammatory conditions such as psoriasis, psoriatic arthritis, or rheumatoid arthritis.

Fc-based fusion proteins represent a major class of therapeutic proteins with seven approved molecules on the market and many others currently under clinical evaluation (1–3). In most cases, therapeutic peptides or proteins are attached to an Fc domain to endow the hybrids with a number of beneficial biological and pharmacological properties (3). Perhaps most importantly, through binding to the neonatal Fc receptor (FcRn), such Fc fusions display IgG-like pharmacokinetic properties (*i.e.* long serum half-life ranging from days to weeks) (4). The very stable Fyn SH3 domain (70 kDa in size and smaller) lacks cysteine residues and, therefore, is monomeric and does not aggregate when天然 occurring domain separating linkers (7) or, the naturally occurring hinge region of an antibody (*i.e.* sequence region between the CH1 and CH2 domains of a full-length antibody), as it is the case for example for the marketed Fc fusion protein etanercept (Enbrel®) (8). In contrast, other proteins of ~70 kDa in size and smaller are typically eliminated rapidly from circulation by renal filtration and have half-lives of a few minutes to a few hours, which can in many cases render them unsuitable for therapeutic applications (5). Beyond half-life extension, Fc fusion can provide several additional benefits such as facilitating expression and secretion of recombinant protein, enabling facile purification by protein A chromatography, binding to Fc receptors and/or complement to support secondary immune functions, improving solubility and stability, and enhancing potency by increasing valency (6).

One of the key variables that has to be addressed when engineering an Fc fusion protein is the choice of the linker length and sequence. Many researchers have used a simple glycine and serine (GGGGS)-containing linker as proposed by a study of naturally occurring domain separating linkers (7) or, the naturally occurring hinge region of an antibody (*i.e.* sequence region between the CH1 and CH2 domains of a full-length antibody), as it is the case for example for the marketed Fc fusion protein etanercept (Enbrel®) (8). In the present article, we show that the linker length plays an important role for the potency of Fc fusion proteins. Using phage display technology (9, 10), we have generated Fynomers inhibiting the activity of the proinflammatory cytokine interleukin 17A (IL-17A). Fynomers are small binding proteins (7 kDa) derived from the human Fyn SH3 domain, which can be engineered to bind to essentially any target of interest with high affinity and specificity (for a review on non-immunoglobulin binding proteins collectively called “scaffolds” (see Refs. 11 and 12). The very stable Fyn SH3 domain (T_{m} ~ 70 °C) is a particularly attractive scaffold for the generation of binding proteins because it (*i*) can be expressed in bacteria in soluble form in high amounts, (*ii*) is monomeric and does not aggregate when stored in solution, (*iii*) lacks cysteine residues and, therefore, can be fused to essentially any other protein without misfolding problems, and (*iv*) is of human origin featuring an amino acid sequence completely conserved in different species from mouse to man and was shown to be non-immunogenic in mammals (10). As other SH3 domains, it is composed of two anti-parallel β-sheets and contains two flexible loops (called RT and...
n-Src loops) to interact with target proteins of interest (Fig. 1A). Recently, we have described the generation of large Fyn SH3 phage display libraries and successful isolation of Fynomers binding to the extra domain B of fibronectin (10), albumin (13), chymase (14), BACE-2 (15), and HER2 (16).

In the present study, we used IL-17A as target because it is a proinflammatory cytokine that has been implied in many autoimmune diseases such as rheumatoid arthritis (17), psoriasis (18), Crohn’s disease (19), multiple sclerosis (20), and lupus erythematosus (21). Several anti-IL-17A inhibitors have been shown in vitro and in vivo to reduce the release of innate immune effectors and are currently being investigated in clinical trials for the treatment of several inflammatory conditions such as rheumatoid arthritis, uveitis, and psoriasis (22–24).

Here, we describe the Fynomer 2C1, which inhibits human IL-17A in vitro with an IC50 value of 2.2 nM. Interestingly, when 2C1 was genetically fused to the Fc part of a human antibody via four different amino acid linkers to yield bivalent binding proteins (each linker differed in length, see Fig. 1C), the 2C1-Fc fusion protein with the longest linker displayed the most potent inhibitory activity, blocking homodimeric IL-17A with an IC50 value of 21 pM, which corresponds to a hundredfold improved IC50 value as compared to the value obtained with monovalent Fynomer 2C1. In contrast, the 2C1-Fc fusion with the shortest linker showed only an ~8-fold improved IC50 value of 260 pM, which can be explained due to increased valency of the Fc fusion. Furthermore, in a mouse model of acute inflammation, we have shown that the most potent 2C1-Fc fusion protein is able to efficiently inhibit IL-17A in vivo. With their suitable biophysical properties, Fynomer-Fc fusion proteins represent new drug candidates for the treatment of IL-17A mediated inflammatory conditions such as psoriasis, psoriatic arthritis, or rheumatoid arthritis.

**EXPERIMENTAL PROCEDURES**

---

**Isolation of Fynomers using Phage Display Libraries**—Using a Fynomer phage display library (10), Fyn SH3-derived binding proteins (Fynomers) specific to IL-17A were isolated using recombinant IL-17A (R&D Systems) as antigen and standard phage display as selection technology (9, 10). After selections, monoclonal bacterial supernatants containing phage were used for phage ELISA (9). Fynomers specifically binding to IL-17A were used as templates for cloning affinity maturation libraries. The process of affinity maturation library generation was essentially the same as described by Schlatter et al. (14) for cloning of the naïve library with randomizations in the RT loop, Src loop, or outside of the loops. After affinity maturation selections, Fynomers were screened for binding to IL-17A by lysate ELISA. Briefly, DNA encoding the Fyn SH3-derived binding proteins were cloned into the bacterial expression vector pQE12 (Qiagen) resulting in C-terminal Myc-His6-tagged constructs as described previously (10). The polypeptides were expressed in the cytosol of *Escherichia coli* bacteria in a 96-well format, and 200 μl of cleared lysate was used for ELISA as described previously (13). The DNA sequence of the specific binders was verified by DNA sequencing (Microsynth).

**Fynomer 2C1 Expression and Purification**—Monomeric Fynomer 2C1 (Fig. 1B) was subcloned into the expression vector pQE12 and therewith fused to a Myc and His6 tag (14). After expression, 2C1 was purified using nickel affinity chromatography (Qiagen) according to the manufacturer’s instructions. Protein size and purity was assessed by SDS-PAGE analysis under non-reducing conditions. 15 μl of a 0.4 mg/ml protein solution was mixed with 5 μl of loading buffer and 15 μl mixture was loaded (NuPAGE® Novex 4–12% Bis-Tris gel, Invitrogen). To determine the protein size, 5-μl full-range rainbow molecular weight marker was used (GE Healthcare). Proteins were allowed to separate in NuPAGE® MES SDS running buffer (Invitrogen), stained with Coomassie staining solution and destained in deionized water.

Protein aggregation state was analyzed using a ÂKTA purifier system (GE Healthcare). 100 μl of a 0.34 mg/ml protein solution was loaded and analyzed on a Superdex 75 10/300 GL column (GE Healthcare).

**Affinity Measurements**—Affinity measurements were performed using a BIACore T200 instrument (GE Healthcare). For the interaction analysis between 2C1 monomer and IL-17A, a Series S CM5 chip (GE Healthcare) was coated with 2000 Resonance Units of IL-17A immobilized using the amine coupling kit (GE Healthcare). The running buffer was PBS containing 0.05% Tween 20. The interactions were measured at a flow of 30 μl/min and injection of different concentrations of Fynomer 2C1 (highest concentration, 100 nM and subsequent 3-fold dilution series). All kinetic data of the interaction were evaluated using the BIACore T200 evaluation software. Similarly, for the interaction analysis between 2C1-Fc fusions and IL-17A, a Series S CM5 chip was coated with 500 Resonance Units of IL-17A immobilized using the amine coupling kit (GE Healthcare). The interactions were measured at a flow of 30 μl/min (PBS containing 0.05% Tween 20) and injection of different concentrations of 2C1-Fc fusions (highest concentration: 50 nM and subsequent 2-fold dilution series). All kinetic data of the interaction were evaluated using the BIACore T200 evaluation software.

**Specificity ELISA**—The target proteins human IL-17A (R&D Systems), human IL-17B (Peprotech), human IL-17C (R&D Systems), human IL-17D (Peprotech), human IL-17E (Peprotech), human IL-17F (AbD Serotec), mouse IL-17A (R&D Systems), rat IL-17A (Akron Biotech), canine IL-17A (R&D Systems), human IL-6 (R&D Systems), human TNF-α (Pierce), ovalbumin (Sigma), bovine serum albumin (BSA, Sigma) cyonmolagus IL-17A (produced in-house), extra domain-B of fibronectin (25) were coated onto MaxiSorp® plates (Nunc) at a concentration of 10 μg/ml. After blocking of residual binding sites with 2% milk in PBS (v/w, Rapilait, Migros, Switzerland), 2C1 monomer was incubated at 80 nM with the antibody 9E10 (Roche Applied Science, 3 μg/ml) in 2% milk in PBS. After washing, the wells were incubated with goat anti-mouse IgG in 2% milk in PBS (1:1000, Sigma, HRP-conjugated). After washing, BM Blue POD (Roche Applied Science) was added as substrate for color development. This reaction was stopped after 5 min with 1 m sulfuric acid (Sigma). Binding to human IL-17C was determined in a separate experiment, indicated by the dashed line (Fig. 2D). For each condition, the reference-subtracted absorbance was calculated by A490 − A650 (reference wavelength) using a SpectraMax® M5e Microplate Reader.
**Anti-IL-17A Fynomer-Fc Fusion Proteins**

(Molecular Devices). The data were imported into Excel (Microsoft), and of the average of the duplicates was calculated.

**Cloning, Expression, Purification, and Quality Control of 2C1-Fc Fusions**—The four different 2C1-Fc fusion proteins were cloned and expressed by the CRO Evitria AG (Schlieren, Switzerland). For purification, 250 ml of cell culture supernatant was applied onto a Mabselect SuRe column (GE Healthcare) using an ÄKTApurifier system (GE Healthcare). The column was washed with 15 column volumes of PBS, pH 7.4, and the protein was eluted using 0.1 M glycine, pH 2.8, collecting 1-ml fractions. After elution, pH was adjusted with 1 M Tris, pH 9. The OD of the fractions was determined using an Infinite M200 pro reader and a NanoQuant plateT (Tecan). The two fractions showing the highest OD were loaded onto an ÄKTApurifier. Preparative size exclusion was performed using a Superdex 200 10/300 GL column (GE Healthcare), and the storage buffer was exchanged to phosphate-buffered saline (PBS, pH 7.4, Invitrogen). The four highest OD fractions were combined and used for further studies. Protein size, purity, and aggregation state was analyzed using an ÄKTApurifier system (GE Healthcare).

**ELISA Competition Experiments**—All four 2C1-Fc fusion proteins were tested in ELISA competition experiments to determine their capabilities to block the interaction between IL-17A and its receptor. Maxisorp plates (Nunc) were coated with commercially available IL-17R-Fc fusion (R&D Systems) overnight at a concentration of 0.6 μg/ml. After blocking with 1% BSA in PBS, different concentrations of the Fc fusions were pre-incubated with 2.5 nM of biotinylated IL-17A (R&D Systems; biotinylated in-house with NHS-PEG4-biotin according to the manufacturer’s instructions (Pierce)); and added to the wells. After washing, the wells were incubated with streptavidin-HRP conjugate (R&D Systems) 1:200 diluted in 1% BSA/PBS. After washing, BM Blue POD (Roche Applied Science) was added as substrate for color development. This reaction was stopped after 5 min with 1 M sulfuric acid (Sigma). For each condition, the reference-subtracted absorbance was calculated by \( A_{450} - A_{650} \) (reference wavelength) using a SpectraMax® Microplate Reader (Molecular Devices). IC50 values were calculated using Prism software (version 5).

**IL-17A Inhibition Cell Assays**—The inhibitory potencies of monomeric 2C1 and all four 2C1-Fc fusion proteins were determined by stimulating normal human dermal fibroblasts with recombinant IL-17A and TNF, as they act in synergy (26). Normal human dermal fibroblast cells (ATCC PCS-201-010) were seeded in 96-well plates (TPP) in fibroblast growth medium (Promocell). The cells were allowed to adhere. Supernatant was exchanged with medium containing IL-17A (34 pm, R&D Systems) and TNF (1.2 pm, Pierce), with and without the anti-IL-17A compounds (2C1, 2C1-Fc fusions, and commercially available IL-17R-Fc (R&D Systems)). As negative control, equal volume of PBS was added to the medium. Additionally, cells were treated with TNF only to mimic IL-6 levels after full IL-17A inhibition (“TNF control”). After 24 h, supernatants were collected, filtered (0.45 μm pore size), and used to determine IL-6 concentrations by ELISA according to the manufacturer’s instructions (DuoSet, R&D Systems). Data points were measured in triplicate. Data indicate a representative result. The percentages of inhibition were plotted, and IC50 values were calculated using Prism software (version 5). The percentage of IL-17A inhibition was determined with the following formula.

\[
\text{Inhibition} (\%) = 100 - \left( \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{TNF control}})}{(\text{OD}_{\text{full stimulation}} - \text{OD}_{\text{TNF control}})} \right) \times 100 \quad (\text{Eq. 1})
\]

**Mouse Pharmacokinetic Study**—2C1L3Fc solution was injected i.v. into five mice (C57BL/6, Charles River) at a dose of 1.5 mg/kg. After the indicated time points, ~20 μl of blood were taken from the vena saphena with the capillary Microvette CB 300 (Sarstedt). The blood samples were centrifuged for 10 min at 9500 × g, and the serum was stored at −20 °C until ELISA analysis was performed. Serum concentration was determined by ELISA using a reference standard of 2C1L3Fc. 50 μl biotinylated IL-17A (30 nm) (R&D Systems, biotinylated using NHS-PEO4-biotin (Pierce) according to the manufacturer’s instructions) was added to streptavidin-coated wells (Reactibind, Pierce) and after blocking with PBS, 4% milk (Rapilait, Migros, Switzerland), 45 μl of PBS, 4% milk, and 5 μl of serum sample were added. After incubation for 1 h and washing, bound Fc fusion proteins were detected with protein A-HRP conjugate (Sigma). Peroxidase activity was detected by addition of QuantaRed enhanced chemifluorescent HRP substrate (Pierce). Fluorescence intensity was measured after 5 to 10 min at 544 nm (excitation) and 590 nm (emission). From the concentrations determined in serum at different time points and the resulting slope \( k \) of the elimination phase (plotted in a semi-logarithmic scale), the half-life of 2C1L3Fc was calculated using the formula \( t_{1/2} = \ln 2/\ln k \).

**Mouse Efficacy Study**—Human IL-17A binds and stimulates the murine receptor resulting in an increase of serum KC-chemokine (CXCL1) levels (31). To test the most potent molecule 2C1L3Fc in vivo, C57BL/6Ncrl mice (Charles River) were injected intravenously with 2C1L3Fc at a dose of 0.5 mg/kg or PBS only. One hour post administration, 3 μl of human IL-17A or PBS was injected subcutaneously. At 2 h after treatment, blood was collected (Microvette CB 300, Sarstedt), and sera were stored frozen until analysis. Five mice were used per treatment group.

**KC levels were determined using the QuantiKine® mouse CXCL1/KC immunoassay (R&D Systems) according to the manufacturer’s instructions. Briefly, 25 μl of sera were mixed with 25 μl of calibrator diluent as described. Then, 50 μl of sample diluent were added. Statistical analyses were performed using GraphPad Prism software (version 6, GraphPad Software) and unpaired t test assuming Gaussian distribution. A p value < 0.05 was considered as statistically significant. All animal studies were approved by the Veterinäraamt des Kantons Zurich (Zurich, Switzerland, license no. 54/2008).

**RESULTS**

**Isolation and Characterization of Fynomer 2C1 Inhibiting IL-17A**—Fynomers specific to human IL-17A were isolated by standard phage display selections (10). After few rounds of panning on biotinylated IL-17A as target, several Fynomers were
identified by phage ELISA. These Fynomers were used as templates for further affinity maturation strategies, introducing new amino acid randomizations in either the RT or Src loop and/or selected amino acids near the loop regions, resulting in the isolation of Fynomer 2C1 (Fig. 1B). Fynomer 2C1 was subcloned into a cytosolic expression vector as a monomer fused to a His6 and Myc tag, expressed and purified using nickel affinity chromatography. 2C1 could be expressed with a high yield of 70 mg/liter under non-optimized conditions in shake flasks, and the resulting protein was 95% pure and monomeric, as determined by size exclusion chromatography (SEC) and SDS-PAGE (Fig. 2, A and B). The affinity of 2C1 toward its target antigen was analyzed by real-time interaction analysis on a BIAcore chip coated with IL-17A, revealing a dissociation constant ($K_d$) of 1.8 nM at the antigen surface density used (Fig. 2C). 2C1 bound human and cynomolgus IL-17A in a specific manner and did not cross-react with any of the other IL-17 family members or IL-17A cytokines from other species, and it did not bind to unrelated proteins as determined by ELISA (Fig. 2D).

The cell-based inhibition assays identified Fynomer 2C1 as a potent IL-17A inhibitor with an IC$_{50}$ value of 2.2 nM (Fig. 2E). Notably, the cell based inhibition assay used both IL-17A and TNF to stimulate the release of IL-6 by normal human dermal fibroblasts as described under “Experimental Procedures.” As expected, 2C1 was able to inhibit specifically IL-17A-induced IL-6 release. As a control, commercially available IL-17-receptor (IL-17R)-Fc fusion protein was used inhibiting IL-17A activity with an IC$_{50}$ value of 0.6 nM (Fig. 2E).

### Generation of Different 2C1-Fc Fusion Proteins Showing Excellent Biophysical Properties

Fynomer 2C1 was genetically fused to the Fc part of a human antibody because of two reasons. First of all, 2C1 carries a molecular mass of ~7 kDa, which is below the renal clearance threshold of 65 kDa, and therefore, it would be rapidly cleared through the kidneys upon injection into animals or humans. The preparation of Fc fusion proteins of otherwise short-lived molecules represents an attractive avenue to prolong the in vivo half-life (6, 27). Second, because IL-17A is a homodimeric protein, we wanted to investigate whether not only valency could be increased but also avidity could be introduced into the binding interaction between 2C1 and IL-17A, i.e. two 2C1 Fynomers binding to one homodimeric IL-17A molecule. It was speculated that increased avidity would result in an increased neutralizing potential of 2C1-Fc fusion proteins. But a priori, it was not clear which linker length between the Fynomer and the Fc part would allow for avid binding to IL-17A. Therefore, four different linkers with variable length were chosen (linker L0, RS; L1, (GGGS)$_1$; L2, (GGGS)$_2$; L3, (GGGS)$_3$) (Fig. 1C). The molecules were expressed in a transient CHO expression system and purified using a two-step method: protein A column followed by preparative SEC. All constructs could be expressed with approximately the same yield (L0, 12.2 mg/liter; L1, 10.3 mg/liter; L2, 22.4 mg/liter; L3, 20.7 mg/liter). The purity, size, and aggregation state were analyzed by SEC, indicating that all purified proteins were >95% pure and monomeric. Affinity measurements and ELISA competition assays with the four Fc fusion proteins showed that the 2C1-Fc fusion with the longest linker had the highest affinity to IL-17A and could block the interaction between IL-17A and its receptor most efficiently (Table 1). Importantly, all four Fc fusion molecules were tested in the bioactivity cell assay as described above, as the conditions of this assay allow to detect low IC$_{50}$ values because IL-17A is used at a very low concentration (34 pm). As expected, all 2C1-Fc constructs showed an increased potency as compared with the monomeric Fynomer 2C1 because of the increased valency (21–260 pm versus 2.2 nm, respectively, Figs. 3 and 2E). Interestingly, only the longer and more flexible linkers of 2C1$_{L2}$Fc and 2C1$_{L3}$Fc improved the IC$_{50}$ values by approximately a factor of forty to one hundred (56 pm and 21 pm, respectively) as compared with the monovalent 2C1 Fynomer, indicating that avidity effects contribute to the increased potency. The Fc fusion proteins with the shorter linkers (2C1$_{L0}$Fc and 2C1$_{L1}$Fc) could only increase the potency.

---

4 The abbreviation used is: SEC, size exclusion chromatography.
by a factor of about eight to nine (260 pM and 232 pM, respectively, Fig. 3).

**DISCUSSION**

IL-17A has been identified as a cytokine that contributes to inflammatory symptoms in autoimmune diseases. IL-17A is found at elevated levels in synovial fluid of patients with rheumatoid arthritis and has been shown to be involved in early rheumatoid arthritis development. IL-17A is a potent inducer of TNF-α and IL-1, the latter being mainly responsible for bone erosion, which has very painful consequences for affected patients (28). Furthermore, inappropriate or excessive production of IL-17A is associated with the pathology of various other diseases and disorders, and therefore, much effort has been invested by the pharmaceutical industry to identify molecules capable of inhibiting the detrimental effects of IL-17A, some of which are currently being tested in clinical trials for different inflammatory conditions (22, 23) such as Crohn’s disease, psoriatic arthritis, ankylosing spondylitis, multiple sclerosis, and polymyalgia rheumatica (www.clinicaltrials.gov).

This study describes a Fynomer derived from the Fyn SH3 domain (10) that inhibits IL-17A in vitro and in vivo with a very high potency. The IL-17A inhibitory Fynomer was isolated from a phage display library with subsequent affinity maturation. The soluble Fynomer has good biophysical properties as determined by SEC and SDS-PAGE (8). Real-time interaction analysis on a BIAcore chip coated with IL-17A was used to determine the dissociation constant (KD). Different concentrations of Fynomer 2C1 (highest concentration, 100 nM and subsequent 3-fold dilution series) were injected revealing a KD value of 1.8 nM at the antigen surface density used. To investigate binding specificity of the monomeric Fynomer 2C1, indicated proteins were coated onto an ELISA plate. 2C1 bound human and cynomolgus (cyno) IL-17A in a specific manner and did not bind to any of the other IL-17 family members or IL-17A cytokines from other species nor to unrelated proteins. Binding to IL-17-C was determined in a separate experiment, indicated by the dashed line. As a positive control for IL-17A inhibition, commercially available IL-17R-Fc fusion was used.

**TABLE 1**

Dissociation constants and IC50 values of the four different 2C1-Fc fusion proteins

| Fusion construct | Affinity (KD) pM | ELISA competition (IC50) pM |
|------------------|-----------------|----------------------------|
| 2C1L0Fc         | 450             | 1809                       |
| 2C1L1Fc         | 357             | 1125                       |
| 2C1L2Fc         | 309             | 842                        |
| 2C1L3Fc         | 276             | 525                        |

In Vivo Experiments with 2C1L3Fc, Pharmacokinetics and IL-17A Inhibition—The in vivo half-life of 2C1L3Fc was determined by measuring serum concentrations at different time points after a single i.v. injection in mice by ELISA. A half-life value of 68 h was calculated from the elimination phase (Fig. 4). Mice treated with human IL-17A upregulate the chemokine KC, which can be measured in serum (31). We here investigated whether 2C1L3Fc can inhibit IL-17A-mediated KC up-regulation in vivo. In the experiment, intravenously injected 2C1L3Fc completely abrogated KC induction by subcutaneously administered human IL-17A (p < 0.0001, Fig. 5), demonstrating the ability of 2C1L3Fc to inhibit human IL-17A in an in vivo setting.

**FIGURE 2.** The Fynomer 2C1 exhibits excellent biophysical properties and a high affinity and specificity to its target protein IL-17A. A, the monomeric Fynomer was expressed in E. coli and purified via a His6 tag affinity chromatography. The resulting protein was 95% pure and monomeric (M), as determined by SEC and SDS-PAGE (8). C, real-time interaction analysis on a BIAcore chip coated with IL-17A was used to determine the dissociation constant (KD). Different concentrations of Fynomer 2C1 (highest concentration, 100 nM and subsequent 3-fold dilution series) were injected revealing a KD value of 1.8 nM at the antigen surface density used. D, to investigate binding specificity of the monomeric Fynomer 2C1, indicated proteins were coated onto an ELISA plate. 2C1 bound human and cynomolgus (cyno) IL-17A in a specific manner and did not bind to any of the other IL-17 family members or IL-17A cytokines from other species nor to unrelated proteins. Binding to 2C1 specifically inhibited IL-17A in a dose-dependent manner. As a positive control for IL-17A inhibition, commercially available IL-17R-Fc fusion was used.
Anti-IL-17A Fynomer-Fc Fusion Proteins

In the study, an anti-IL-17A Fynomer-Fc fusion protein was developed to inhibit IL-17A with a potency of 0.6 nM. The fusion of an Fc portion to a homodimeric ligand was expected to have the dual benefits of both an increase in pharmacokinetic through avid binding to the IL-17A homodimer, and the introduction of short linkers (either the amino acids RS or GGGGS) was not sufficient to allow for a highly avid binding mode. The expected increase in potency did not increase significantly (IC_{50} values of 2.2 nM, 260 pM, or 232 pM for monomeric 2C1 or 2C1-Fc fusions with RS or GGGGS linkers, respectively). Increased flexibility due to longer linkers of 10 or 15 amino acids, (GGGGS)_{2} and (GGGGS)_{3}, led finally to an up to 105-fold increase in activity, resulting in a highly potent IL-17A inhibitor (2C1L3Fc) with an IC_{50} value of 21 pM. Importantly, in a murine acute inflammation model, 2C1L3Fc was shown to inhibit IL-17A in vivo. These data exemplify an important aspect of Fc fusion biotechnology, e.g., the ability to engineer a drug candidate for needed potency and valency. In addition, we expect 2C1L3Fc to be a selective inhibitor of the IL-17A/A homodimer with a preference over the IL-17A/F heterodimer, due to the avid binding mode toward the IL-17A/A homodimer.

In summary, we have described the isolation of an anti-IL-17A Fynomer, and by using the inherent flexibility of the technology platform, we have been able to use Fc fusion formatting to produce one of the most potent IL-17A inhibitors described to date. A systematic study of increased linker length between the binding Fynomer and the Fc region showed that a critical length of linker is required to provide full flexibility to allow simultaneous binding to both chains of the IL-17A homodimer. The encouraging results described herein are highly promising for future preclinical and clinical development of 2C1L3Fc for the treatment of different inflammatory conditions.

Acknowledgment—We thank Dr. Frank Bootz for the invaluable technical assistance for all in vivo studies.

REFERENCES

1. Beck, A., and Reichert, J. M. (2011) Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies. MAbs 3, 415–416
2. Walsh, G. (2010) Biopharmaceutical benchmarks 2010. Nat. Biotechnol. 28, 917–924
3. Czajkowsky, D. M., Hu, J., Shao, Z., and Pleass, R. J. (2012) Fc-fusion proteins: new developments and future perspectives. EMBO Mol. Med. 4, 1015–1028
4. Huang, C. (2009) Receptor-Fc fusion therapeutics, traps, and MIMETIC technology. Curr. Opin. Biotechnol. 20, 692–699
5. Kontermann, R. E. (2009) Strategies to extend plasma half-lives of recombinant antibodies. BioDrugs 23, 93–109
6. Carter, P. J. (2011) Introduction to current and future protein therapeutics: a protein engineering perspective. Exp. Cell Res. 317, 1261–1269
7. Argos, P. (1990) An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion. J.

FIGURE 3. 2C1-Fc fusion molecules show linker-length dependent IL-17A inhibition in vitro. 2C1 was genetically fused to the Fc-part of a human IgG1 using four linkers of variable length (L0 – L3, see Fig. 1C). The Fc-fusion molecules were tested in a cell assay after stimulation with IL-17A and TNF. The Fynomer-Fc fusion proteins with longer and more flexible linkers, 2C1L2Fc and 2C1L3Fc, showed most potent inhibition of IL-17A.

FIGURE 4. Serum concentrations of 2C1L3Fc in mice. Serum concentrations in serum are plotted in a semi-logarithmic scale versus time after a single intravenous injection in mice.

FIGURE 5. IL-17A inhibition in vivo by 2C1L3Fc. Intravenously injected 2C1L3Fc completely inhibited IL-17A-induced KC up-regulation in mice. Mean KC levels of five mice per group are shown (± S.D.). *** p < 0.0001. ns, not significant.
Anti-IL-17A Fynomer-Fc Fusion Proteins

Mol. Biol. 211, 943–958

Mohler, K. M., Torrance, D. S., Smith, C. A., Goodwin, R. G., Stremler, K. E., Funk, V. P., Madani, H., and Widmer, M. B. (1993) Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. J. Immunol. 151, 1548–1561

Viti, F., Nilsson, F., Demartis, S., Huber, A., and Neri, D. (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. Methods Enzymol. 326, 480–505

Grabulovski, D., Kaspar, M., and Neri, D. (2007) A novel, non-immunogenic Fyn SH3-derived binding protein with tumor vascular targeting properties. J. Biol. Chem. 282, 3196–3204

Bertschinger, J., Grabulovski, D., and Neri, D. (2007) Selection of single domain binding proteins by covalent DNA display. Protein Eng. Des. Sel. 20, 57–68

Schlatter, D., Brack, S., Banner, D. W., Batey, S., Benz, J., Bertschinger, J., Huber, W., Joseph, C., Rufer, A., van der Klooster, A., Weber, M., Grabulovski, D., and Hennig, M. (2012) Generation, characterization and structural data of chymase binding proteins based on the human Fyn kinase SH3 domain. MAb 4, 497–508

Banger, D. W., Gsell, B., Benz, J., Bertschinger, J., Burger, D., Brack, S., Cuppuleri, S., Debulpaep, M., Gast, A., Grabulovski, D., Hennig, M., Hilpert, H., Huber, W., Kuglstatter, A., Kusznir, E., Laeremans, T., Matile, H., Miscenic, C., Rufer, A. C., Schlatter, D., Steyaert, J., Stihle, M., Thoma, R., Weber, M., and Ruf, A. (2013) Mapping the conformational space accessible to BACE2 using surface mutants and co-crystals with Fab fragments, Fynomers and Xapernones. Acta Crystallogr. D Biol. Crystallogr. 69, 1124–1137

Mourlane, F., Toller, I., Brack, S., Grabulovski, D., and Bertschinger-Ehler, J. (2012) Bispecific Fynomer-antibody fusion proteins targeting two epitopes on HER2. J. Clin. Oncol. 30, 2575

Ziolekowska, M., Koc, A., Luszczykiewicz, G., Ksiezopolska-Pietrzak, K., Klimeczek, E., Chwalińska-Sadowska, H., and Maslinski, W. (2000) High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. J. Immunol. 164, 2832–2838

Arican, O., Aral, M., Sasmaz, S., and Ciragil, P. (2005) Serum levels of IL-17A in patients with active psoriasis and correlation with disease severity. Mediators Inflamm. 2005, 273–279

Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., Bamba, T., and Fujiyama, Y. (2003) Increased expression of interleukin-17 in inflammatory bowel disease. Gut 52, 65–70

Brucklacher-Waldert, V., Steinbach, K., Liozov, M., Kolster, M., Hölscher, C., and Tolosa, E. (2009) Phenotypical characterization of human Th17 cells unambiguously identified by surface IL-17A expression. J. Immunol. 183, 5494–5501

Nalbandian, A., Crispin, J. C., and Tsokos, G. C. (2009) Interleukin-17 and systemic lupus erythematosus: current concepts. Clin. Exp. Immunol. 157, 209–215

Genovese, M. C., Van den Bosch, F., Roberson, S. A., Bojin, S., Biagini, I. M., Ryan, P., and Sloan-Lancaster, J. (2010) LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study. Arthritis Rheum. 62, 929–939

Hueber, W., Patel, D. D., Dryja, T., Wright, A. M., Koroleva, I., Brin, G., Antoni, C., Draelos, Z., Gold, M. H., Psoriasis Study Group, Durez, P., Tak, P. P., Gomez-Reino, J. J., Rheumatoid Arthritis Study Group, Foster, C. S., Kim, R. Y., Samson, C. M., Falk, N. S., Chiu, D. S., Callanan, D., Nguyen, Q. D., Uvetis Study Group, Rose, K., Haider, A., and Di Padova, F. (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. Sci. Transl. Med. 2, 52ra72

Leonardi, C., Matheson, R., Zachariae, C., Cameron, G., Li, L., Edson-Heredia, E., Braun, D., and Banerjee, S. (2012) Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. N. Engl. J. Med. 366, 1190–1199

Carmemolla, B., Neri, D., Castellani, P., Leprini, A., Neri, G., Pini, A., Wintner, G., and Zardi, L. (1996) Phage antibodies with pan-species recognition of the oncofoetal angiogenesis marker fibronectin ED-B domain. Int. J. Cancer 68, 397–405

Fossiez, F., Djossou, O., Chomarat, P., Flores-Romo, L., Ait-Yahia, S., Maat, C., Pin, J., Garrone, P., Garcia, E., Saeld, S., Blanchard, D., Gallard, C., Das Mahapatra, B., Rouvier, E., Golstein, P., Banchereau, J., and Lebecque, S. (1996) T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J. Exp. Med. 183, 2593–2603

Suzuki, T., Ishii-Watabe, A., Tada, M., Kobayashi, T., Kanayasu-Toyoda, T., Kavanishi, T., and Yamaguchi, T. (2010) Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. J. Immunol. 184, 1968–1976

Lubberts, E. (2008) IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? Cytokine 41, 84–91

Wozniak-Knopp, G., Bartl, S., Mostageer, M., Woisetschläger, M., Antes, B., Ettl, K., Weberhofer, G., Wiederkum, S., Himmler, G., Mudde, G. C., and Rüker, F. (2010) Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng. Des. Sel. 23, 289–297

Coppieters, K., Dreier, T., Silence, K., de Haard, H., Lauwereys, M., Antes, B., Ettl, K., M., Weberhofer, G., Wiederkum, S., Himmler, G., Mudde, G. C., and Rüker, F. (2010) Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng. Des. Sel. 23, 289–297

Coppieters, K., Dreier, T., Silence, K., de Haard, H., Lauwereys, M., Antes, B., Ettl, K., M., Weberhofer, G., Wiederkum, S., Himmler, G., Mudde, G. C., and Rüker, F. (2010) Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng. Des. Sel. 23, 289–297