Properties of 7ND–CCL2 are modulated upon fusion to Fc

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7ND, a truncated version of the chemokine MCP-1/CCL2 lacking amino acids 2–8, is a potent antagonist of CCR2. In contrast to CCL2, 7ND is an obligate monomer. Similar to other chemokines, the in vivo half-life of 7ND is very short and its use as an antagonist in disease models is thus limited. We therefore constructed a 7ND-Fc fusion protein to extend the half-life of 7ND and overcome its limitations as a potential therapeutic antagonist. When we tested the properties of the fusion molecule in vitro, we found to our surprise that 7ND-Fc, in contrast to 7ND, produced a distinct, albeit small, chemotactic response in THP-1 cells, and a robust chemotactic response in L1.2 cells stably transfected with CCR2. To test whether this unexpected observation might be due to the bivalency of 7ND-Fc stemming from the dimeric nature of Fc fusions, we produced a heterodimeric Fc fusion which displays only one 7ND moiety, using a technology called strand exchange of engineered CH3 domains (SEED). The monovalent construct had properties equivalent to the parent 7ND. Furthermore, partial agonist activity appears to depend on receptor density as well as the signaling pathway examined. However, we were able to show that 7ND-Fc, but not 7ND alone, has antagonistic activity in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis.

Keywords: anti-inflammatory/chemokine receptor antagonist/Fc fusion protein

Introduction

CCR2 is one of the major chemokine receptors expressed on circulating monocytes and is a marker for the subset of CD14⁺CD16⁻ inflammatory monocytes (Tacke and Randolph, 2006). CCL2/MCP-1 is the principal ligand for CCR2 because mice in which it has been deleted have phenotypes that closely resemble those of CCR2⁻/− mice (Boring et al., 1997; Lu et al., 1998). CCR2 is also expressed on memory T-lymphocytes, dendritic cells, NK cells and microglia (Sozzani et al., 1995). The CCL2–CCR2 interaction is thought to be responsible for the migration of these cells to sites of infection and inflammation and is thus implicated in many human diseases. In agreement with these data, it has been described that full-length CCL2 enhances arthritis (Gong et al., 1997).

The action of chemokines has often been described as redundant. However, despite the existence of many other CC chemokines that attract monocytes, CCL2⁻/− mice are unable to recruit monocytes 72 h after intraperitoneal (i.p.) injection of thioglycollate, showing that loss of CCL2 alone is sufficient to impair monocyte trafficking (Lu et al., 1998).

There is evidence that the CCL2–CCR2 axis plays a key role in many inflammatory and autoimmune diseases (Charo and Ransohoff, 2006), as well as in disorders of the central nervous system (CNS) (Ransohoff, 1997; Huang et al., 2000) and in cancer (Conti and Rollins, 2004). For example, CCL2 has been linked with inflammatory autoimmune-related kidney disease, such as lupus nephritis (Kulkarni and Anders, 2008), glomerulonephritis and diabetic nephropathy. Furthermore, it has been linked to Crohn’s disease and ulcerative colitis, where upregulation of CCL2 was shown to correlate with increased disease activity (Banks et al., 2003). Elevated expression of CCL2 is also seen in asthmatic patients, where the expression level correlates with the severity of the symptoms (Hsieh et al., 1996).

Several studies have described the expression of CCL2 in the CNS of patients with multiple sclerosis (MS) (McManus et al., 1998; Van Der Voom et al., 1999) and CCL2 expression levels compare with clinical severity and attacks in relapsing experimental autoimmune encephalomyelitis (EAE), a common mouse model for MS (Kennedy et al., 1998). The CCR2–CCL2 axis is further validated by neutralization of CCL2 in EAE (Fife et al., 2001) and the phenotype of the CCR2⁻/− mice which are resistant to myelin oligodendrocyte glycoprotein (MOG)-induced EAE (Izikson et al., 2000). For these reasons, CCR2 has been considered as an interesting target for the therapy of these diseases.

Evidence has accumulated that CCR2 may be involved in rheumatoid arthritis (RA), although there are several ambiguities. However, so far three clinical trials of CCR2 inhibitors in RA have failed suggesting that it might not therefore be the most relevant chemokine receptor in this disease (Proudfoot, 2008).

7ND is a mutant of CCL2 that lacks the N-terminal amino acids 2–8 and that binds to CCR2 with almost the same affinity as the wild-type CCL2 (Zhang et al., 1994) yet it is unable to induce chemotaxis or Ca²⁺-mobilization in vitro and is therefore a potent CCR2 antagonist. 7ND gene therapy has been shown to reduce symptoms in a wide variety of disease models. For example, 7ND gene therapy in experimental autoimmune myocarditis reduced disease severity and prevalence (Goser et al., 2005). In cancer, 7ND gene therapy reduced the recruitment of tumor-associated macrophages as well as tumor angiogenesis and growth of malignant melanoma (Koga et al., 2008).
7ND, like other chemokines and small proteins, has a short half-life in vivo, and as a result the therapeutic applicability of the protein is limited. An established method to prolong the half-life of proteins is to fuse them to the Fc portion of antibodies. Examples are Etanercept (a TNFα-receptor-Fc fusion protein), Alefacept (a fusion protein of LFA-3 with an Fc) and Abatacept (hCTLA-4 fused to Fc) (Huang, 2009).

We therefore constructed a 7ND-Fc fusion protein to improve the half-life of 7ND in vivo for testing in disease models. Characterization in vitro demonstrated that the fusion of the antagonist protein to the Fc resulted in an unexpected gain of activities. These new activities were not detected with a monovalent 7ND-SEED fusion, suggesting that bivalency was responsible for the observed gain of function. Despite the partial agonist activity of 7ND-Fc, it was able to inhibit chemotaxis in vitro which translated into delayed onset of disease in an EAE model.

Materials and methods

Reagents

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich (Buchs, Switzerland). Media and cell culture reagents were from Invitrogen (Lucerne, Switzerland). Enzymes were purchased from New England Biolabs (Frankfurt a. M., Germany), and chromatographic material was from GE Healthcare (Glattbrugg, Switzerland).

Generation of 7ND-Fc

A plasmid coding for the CCL2 mutant P8A (Paavola et al., 1998), fused to an IgG1-Fc tail, was used as a starting point for site-directed mutagenesis to delete the bases coding for amino acids 2–9 of P8A, in order to generate 7ND-Fc. The primers used for this operation were the following (position of the deletion indicated by a star): 5'-CAGGGGCTCGCT CAG*GTCACCTGCTGTTATAACTTC-3' (sense) and 5'-GAAGCTCTCTGCCGCCC-3' (antisense). The following primers were used for this amplification: 5'-CATTCGCCAGGGGCTCGCT GACAAAACCTCAACATGCC-3' (sense) and 5'-CAAGA AAGCTGGGTTCGCGCAAGGAGATGGACTTCTGCCG-3' (antisense). The signal peptide was amplified separately, adding an attB1 site at the 5'-end, using the following primers: 5'-GACGGCTTCGCTGACAGGAGATGGACTTCTGCCG-3' (sense) and 5'-GGGATGGTGTAGCTTTTG TCAGCGAGGCCCCGAGGAATGAAG-3' (antisense). The two fragments were assembled by PCR using the antisense primer of the first amplification reaction and the sense primer from the amplification reaction of the signal peptide.

Generation of 7ND-SEED

A plasmid containing the [GA] CH3 SEED-domain (Davis et al., 2010) was amplified using a primer providing an overlap with the CH2-domain, 5'-AACATCCTCGAGGAAGTTTAACTGG-3' (sense), and a second primer adding an attB site for gateway cloning, 5'-CGCCCTTC-3' (antisense). 7ND fused to the CH2-domain via a hinge region was amplified from the 7ND-Fc coding sequence with 250 ml of a solution of 1 × 10⁶ cells was prepared and transfeected with 50 ml of a transfection mix containing 500 µg DNA (the ratio of AG-SEED plasmid to the plasmid coding for the GA SEED strand fused to 7ND was 3:2) and 1 mg polyethylenimine in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Invitrogen) supplemented with 1% fetal calf serum (FCS) and 4 ml/l insulin-transferrin-selenium A (ITSx), which had been pre-incubated for 10 min. The transfected cells were incubated at 37°C for 90 min with gentle stirring and then completed with 250 ml of Freestyle serum-free medium. The cells were then incubated at 37°C in a 5% CO₂-atmosphere for 6 days, with gentle stirring.

Cells were harvested by centrifugation on Day 6 after transfection. The supernatant was filtered through a 0.22 µm filter and applied to a protein A column (Mab select sure, GE Healthcare). After loading, the column was washed with 0.1 M Tris-HCl, pH 7.2 and protein was eluted with 0.1 M glycine, pH 2.7, in 5 ml fractions containing 500 µl 1 M Tris-HCl buffer, pH 8.0, in order to immediately neutralize the protein. The fractions containing the protein were pooled and subjected to gel filtration on a Superdex-200 column with phosphate-buffered saline (PBS) as the running buffer. The proteins were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis, UV-spectroscopy and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS).

7ND was expressed in Escherichia coli. The cells were broken by two rounds of treatment with a French Press at
In vivo chemotaxis

Chemotaxis assays were carried out using 96-well microplates (ChemoTx, Neuro Probe, Gaithersburg, MD, USA) and either THP-1 cells or L1.2 cells expressing CCR2. THP-1 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM l-glutamine and penicillin/streptomycin (50 units/ml each). L1.2 cells were cultured in DMEM/F12 medium containing 5% heat-inactivated FCS, 2 mM l-glutamine and 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and 0.8 mg/ml geneticin. The L1.2 cells were cultured at 0.5 × 10^6 cells/ml and activated with 5 mM sodium butyrate one day before use in chemotaxis assays. On the day of the chemotaxis assay, the cells were collected by centrifugation (600 g) and resuspended at a concentration of 1.0 × 10^7/ml (for THP-1), or 3.0 × 10^7/ml (for L1.2) in RPMI 1640 medium containing 5% heat-inactivated FCS without phenol red. A dilution series of the proteins was prepared covering the range of 10^{-6}–10^{-12} M in RPMI medium without phenol red. Thirty-three microliters of each dilution were placed in the wells of the lower plate. A filter membrane (5 μm pore size for THP-1, 8 μm pore size for L1.2 cells) was placed over the plate. Twenty microliters of the cell solution were deposited on top of the membrane of each well (2.0 × 10^4 or 6.0 × 10^4 cells for THP-1 or L1.2, respectively). The chamber was incubated for 2 h for THP-1 or 4 h for L1.2 at 37°C under 5% CO₂. Experiments for the inhibition of chemotaxis were performed accordingly with both the constant concentration of CCL2 (1 nM) and the concentrations of the antagonist, ranging from 10^{-12} to 10^{-6} M, in the lower wells. The cell suspension was then carefully removed by washing with PBS and the filter was removed. The migrated cells in the lower wells were transferred to a black plate using a 96-well funnel adaptor and frozen for at least 2 h at −80°C. The plate was then thawed and a solution of CyQuant dye/cell-lysis buffer mix (200 μl; Invitrogen) was added to each well. Fluorescence was counted using a Victor2 Wallac plate reader and the data were analyzed using Graphpad Prism software.

Fluorescence-activated cell sorting analysis of receptor expression levels

1 × 10^5 cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% FCS and 0.01% sodium azide) and incubated on ice with 100 μl of 100-fold diluted anti-CCR2-PE antibody (FAB151P, R&D Systems, Wiesbaden, Germany) or an IgG2b-PE isotype control for 30 min. The cells were then washed three times with ice-cold FACS buffer and analyzed by flow cytometry using a Facsiscalibur with FlowJo analysis software.

Ca²⁺ mobilization

The assay was performed on THP-1, L1.2/CCR2 or CHO/CCR2 cells. L1.2 cells were split to a concentration of 0.5 ×
10⁶ cells/ml and activated with 5 mM sodium butyrate one day before use in Ca²⁺-mobilization assays. THP-1 and L1.2 cells were incubated with fluo-4 NW dye (Invitrogen) for 1 h at 37°C, transferred to a black 384-well plate with transparent bottom at 3.3 × 10⁶ cells/ml (15 μl/well) and briefly centrifuged in order to obtain a cell layer at the bottom of the plates. CHO cells were cultured at 15 000 cells per well one day before the assay. On the day of the assay, the supernatant was removed, 15 μl of fluo-4 NW dye were added to each well and the cells were incubated for 1 h at 37°C. Proteins to be tested were prepared in HBSS supplemented with 15 mM HEPES and 8 mM EGTA, pH 7.4, at a concentration of 400, 40, 4 or 0.4 nM to obtain final concentrations of 100, 10, 1 or 0.1 nM in the assay. Five microliters of the protein solution were added to 15 μl of the dye-loaded cells and Ca²⁺ mobilization was measured every second for 5 min using a Calcium Imaging Plate Reader FDSS7000 (Hamamatsu Photonics).

**ERK-phosphorylation**

The experiment was performed using either L1.2/CCR2 or CHO/CCR2 cells. L1.2 cells were split to a concentration of 0.5 × 10⁶ cells/ml and activated with 5 mM sodium butyrate one day before use in the assay. CHO cells were detached by trypsinization. All cells were counted, washed once with starvation medium (RPMI medium without FCS) and resuspended in starvation medium at 5 × 10⁶ cells/ml. The cells were then incubated for 3 h at 37°C in a 5% CO₂-atmosphere. For stimulation, 5 μl of the protein at a concentration of 10 μM was added to 500 μl of the cells, and incubated for 15 s, 1 min or 3 min in a 37°C water bath. The activation was stopped by quick centrifugation, removal of the supernatant, addition of 150 μl of 2× Tris-glycine SDS sample buffer supplemented with DTT and brief sonication. The samples were then boiled at 95°C for 5 min, electrophoresed on an SDS gel and subjected to western blot analysis, using rabbit anti-phosphor p44/42 MAPK (ERK1/2) as the first antibody (#9101, Cell Signaling through BioConcept, Allschwil, Switzerland) and swine anti-rabbit-HRP as the secondary antibody (P0217, DAKO, Baar, Switzerland).

**Receptor internalization assay**

Human peripheral blood mononuclear cells (PBMCs) (1 × 10⁷ cells) were pre-incubated in RPMI medium containing 10% FCS at either 4 or 37°C, and then incubated at the same temperature with various concentrations of chemokine reagents (0–375 μM for CCL2 and 7ND, 0–88 μM for 7ND-Fc and 0–49 μM for 7ND-SEED, as indicated in Fig. 9) in a total volume of 200 μl. After 30 min the cells were placed on ice for 15 min, washed once with 200 μl of ice-cold PBS containing 1% BSA and incubated with anti-CCR2 antibody (Doc-3, M. Mack) at 4°C for 45 min. The cells were then washed three times and incubated with secondary antibody (R0439, DakoCytomation, Hamburg, Germany) for 30 min at 4°C. After two washing steps, receptor expression on the cell surface was detected by flow cytometry using a Facsscalibur with CellQuest analysis software.

For the analysis of the results, the response obtained for incubation with medium only was set to 100% implying that there was no internalization, and the isotype control was set to 0% implying that there is no signal for CCR2 expression on the surface, resembling 100% receptor internalization.

**Experimental autoimmune encephalomyelitis**

Ten-week-old C57BL6/J mice were anesthetized and immunized with complete Freund’s adjuvant emulsified with MOG peptide 35–55 (NeoMPS, Strasbourg, France) at the dose of 200 μg/mouse plus 250 μg Mycobacterium tuberculosis/mouse (H37 Ra, Difco, Basel, Switzerland) diluted in PBS. Mice received a concomitant i.p. injection of Pertussis toxin (Alexis Biochemicals, Lausen, Switzerland) at a dose of 300 ng/mouse. Challenge with Pertussis toxin was repeated 48 h later. Sham mice were treated similarly except that the emulsion used for their immunization did not contain MOG. Mice were treated daily from Day 7 until the end of the experiment by an s.c. injection of vehicle (0.02% BSA in PBS), 7ND or 7ND-Fc at the indicated doses. Clinical scores were sums of individual scorings assigned to the tail (0–2), hindlimbs (0–4), forelimbs (0–2) and signs of incontinence (0–1), adding each component to a maximal total clinical score value of 9. More precisely, for the tail the scores were given as follows: no signs of disease = 0, floppy tail = 1, complete tail paralysis = 2. For the hind limbs: flip test positive = +1; flip test positive + mouse tumbles when walking on the cage grid = +2, mice hangs only with forelimbs placed under the cage grid (i.e. cannot grasp with hindlimbs) = +3 and paralysis = +4. For symptoms in the forelimbs the following scores were given: weakness = +1 or forelimb paralysis = +2. In the presence of incontinence an additional 1 point was added to the final score. No deaths were observed in the reported experiment. Daily clinical score data were analyzed by Kruskal–Wallis followed by Dunn’s multiple comparison test. Curves for the incidence of disease were compared using log-rank (Mantel–Cox test).

**In vivo depletion of CCR2⁺ cells by ADCC**

Female C57BL6/J mice were treated s.c. with depleting rat anti-CCR2 antibody [M. Mack, 0.5 mg/kg (MC-21, Mack et al., 2001)] or with 7ND-Fc (0.1, 0.5 or 2.5 mg/kg). Mice were sacrificed 24 h later and the blood was collected by cardiac puncture. Total white blood cells were counted using a BD Coulter cell counter after lysis of blood erythrocytes by two cycles of incubation in lysis solution for 3 min at 37°C and washed twice. For FACS analysis cells were incubated with 10% heat-inactivated mouse serum and stained for 30 min with a mix containing CD11b-APC (Becton Dickinson, Allschwil, Switzerland) and Ly6C- and -G-FITC (Becton Dickinson, Allschwil, Switzerland) and washed twice. Stained cells were analyzed using a Facsscalibur (BD Biosciences).

**Results**

**Cloning and purification of 7ND-Fc and 7ND-SEED**

In order to improve the pharmacokinetic properties of the CCR2 antagonist 7ND, an IgG1-Fc tail was fused to the C-terminus of the protein. Furthermore, we constructed a heterodimeric protein with an IgA/IgG chimeric Fc displaying only one 7ND moiety, called 7ND-SEED (Davis et al., 2010) (Fig. 1). After expression of the proteins in HEK 293 EBNA cells and purification of the supernatant by protein A
followed by size exclusion chromatography, the proteins were found to be >95% pure. No trace of the homodimers was detected for the 7ND-SEED by SDS-polyacrylamide gel electrophoresis or matrix-assisted laser desorption ionization-time-of-flight analysis. As expected, MALDI-MS analysis showed a slightly higher mass ($m/z = 71,384$ Da and 63,144 Da for 7ND-Fc and 7ND-SEED, respectively) than theoretically calculated ($m/z = 68,226$ Da and 59,641 Da, respectively), probably due to glycosylation of both the Fc and CCL2 (Ruggiero et al., 2003).

**Pharmacokinetic properties of 7ND and 7ND-Fc**

To determine whether the fusion of the Fc to 7ND had the desired effect on the half-life of the protein, 2 mg/kg of either 7ND or 7ND-Fc was injected s.c. into female C57BL-6 mice. In the case of 7ND the half-life was determined to be 1.75 h and 7ND could no longer be detected in the serum after 24 h. The concentration of 7ND-Fc on the other hand was still at its maximal value after 48 h (Fig. 2).

**Equilibrium competition receptor binding**

The binding affinity of 7ND, 7ND-Fc and 7ND-SEED for the CCR2 receptor was determined using an SPA with CHO membranes expressing CCR2 (Fig. 3). The IC$_{50}$ for 7ND is $1.26 \pm 1.0$ nM, which is very similar to that obtained for CCL2 ($1.10 \pm 0.7$ nM). The fusion of Fc to 7ND increases the binding affinity compared with 7ND, and we determined an IC$_{50}$ of $0.06 \pm 0.06$ nM. This could be due to the fact that 7ND-Fc presents two 7ND molecules and the binding may therefore be enhanced through an avidity effect. For the 7ND-SEED the IC$_{50}$ was found to be $7.29 \pm 2.6$ nM.

**In vitro chemotaxis and inhibition of chemotaxis**

We tested the ability of 7ND and its constructs to inhibit chemotaxis induced by wild-type CCL2 in view of testing them in a disease model. 7ND as well as the fusion proteins 7ND-Fc and 7ND-SEED were able to inhibit the chemotaxis of L1.2 cells stably transfected with CCR2 induced by 1 nM CCL2, with IC$_{50}$ values of $20.7 \pm 16.9$, $12.6 \pm 18.4$ and $46.2 \pm 43.9$ nM, respectively (Fig. 4). However, in order to control for partial agonist activity we performed chemotaxis experiments with THP-1 and L1.2/CCR2 transfectants. We observed that, unexpectedly, 7ND-Fc provoked a small response in THP-1 cells, and robust cell recruitment of L1.2/
CCR2 transfected cells. The magnitude of the response appears to correlate with receptor density, as THP-1 cells express significantly lower levels of CCR2 than the stably transfected L1.2 cells (Figs 5B and 6B). While no response was observed for 7ND in THP-1 cells, a response was observed for this protein using the L1.2 cells, although at a significantly higher concentration (100 nM) than for CCL2 (1 nM), and reaching only \( \sim 50\% \) of the maximal response for CCL2. The maximum for 7ND-Fc was found at similar concentrations as for CCL2, but in contrast to the chemotaxis observed for THP-1 cells, here the response reached \( \sim 50\% \) of the intensity of CCL2 (Fig. 6A). To confirm that this response is not provoked by the Fc part of 7ND-Fc we tested a different chemokine-Fc fusion, SDF-Fc. In this case no chemotaxis was observed.

The observation that 7ND-Fc and 7ND have significantly different behavior was quite unexpected. We postulated that this was likely due to the dimeric nature of the protein imposed by the Fc portion. To investigate this possibility we constructed the heterodimeric 7ND-SEED, containing an engineered Fc that displays only one 7ND moiety. The 7ND-SEED construct did not induce chemotaxis of THP-1 cells, as is the case for the parent 7ND (Fig. 5A), while a similar response was observed for 7ND and 7ND-SEED with L1.2/CCR2 cells (Fig. 6A).

\[ Ca^{2+} \] mobilization

To investigate the signaling pathways that are activated by 7ND-Fc in more detail, we performed \([Ca^{2+}] \) mobilization assays. For these we again used THP-1 and L1.2/CCR2 cells, as well as an adherent cell line, CHO cells expressing CCR2. The cells were loaded with a \([Ca^{2+}] \)-sensitive dye and then stimulated with CCL2, 7ND, 7ND-Fc or 7ND-SEED. We found that in all cells only CCL2 was able to induce a \([Ca^{2+}] \) response, while neither 7ND alone nor 7ND fused to Fc or SEED were able to mobilize intracellular \([Ca^{2+}] \) (Fig. 7).

\[ ERK \] phosphorylation

To explore the activation of another signaling pathway by 7ND and its two fusion constructs, we performed \[ ERK \]-phosphorylation studies using CHO/CCR2 and L1.2/CCR2 cell lines. \[ ERK \]-phosphorylation was visualized by western blot analysis using an anti-ERK1/2 antibody, which reveals two bands at 42 and 44 kDa (Supplementary Fig. S1). In all cases we found that the lower band at 42 kDa corresponding to ERK2 was more intense than the upper band at 44 kDa. The intensity of this band was therefore used for quantitative analysis (Fig. 8), although integration of the 44 kDa band led to the same quantitative profile (data not shown). While 7ND and 7ND-SEED gave no \[ ERK \]-phosphorylation in CHO/CCR2 cells compared with

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**Fig. 6.** Chemotaxis assay with L1.2/CCR2 cells. (A) A chemotactic response is observed for 7ND-Fc that reaches \( \sim 50\% \) of the maximal CCL2-induced response. Chemotaxis is also observed for 7ND and 7ND-SEED at high concentrations. (B) FACS analysis confirming expression of CCR2 on the L1.2 cells.

**Fig. 7.** \([Ca^{2+}] \) mobilization assays. \([Ca^{2+}] \) mobilization assays were performed using (A) CHO cells stably expressing CCR2, (B) THP-1 or (C) L1.2 cells stably expressing CCR2. Neither 7ND nor 7ND-Fc nor 7ND-SEED induced \([Ca^{2+}] \) mobilization in any of the three cell lines tested.
the control without stimulation, 7ND-Fc shows low but distinct ERK-phosphorylation after 3 min of stimulation with 7ND-Fc, reaching an intensity of $\sim 50\%$ compared with stimulation with CCL2 after 3 min (Fig. 8A). On the other hand, in L1.2/CCR2 cells, the intensity of ERK-phosphorylation after stimulation with 7ND-Fc is about the same intensity as for stimulation with CCL2 after 1 min, while after 3 min the signal is even more intense than for CCL2 (Fig. 8B). In contrast to the reaction in CHO cells, in L1.2 cells we observed some ERK-phosphorylation for the stimulation with 7ND and 7ND-SEED after 3 min. This observation parallels the results obtained for chemotaxis in L1.2 cells and may be due to high surface expression of the CCR2 receptor.

**Receptor internalization**

We examined whether stimulation of CCR2-expressing cells with the 7ND constructs leads to receptor internalization. To this end human PBMCs were stimulated with increasing concentrations of CCL2, 7ND, 7ND-Fc or 7ND-SEED at 37°C for 30 min. The receptor expression was subsequently evaluated by FACS analysis. Cells that had not been stimulated were defined to express 100% receptor on the surface, whereas staining with an isotype antibody was used to define 0% receptor on the surface. The surface expression of CCR2 following stimulation was calculated accordingly. CCL2 stimulation led, as expected, to a dose-dependent internalization of CCR2 (Fig. 9). 7ND-Fc as well as 7ND-SEED internalized the receptor in a dose-dependent manner, while no internalization was observed for 7ND. When the receptor internalization was analyzed at 4°C neither CCL2 nor any of the modified versions induced receptor internalization (data not shown).

**Experimental autoimmune encephalomyelitis**

We tested the 7ND-FC protein and 7ND alone in MOG-induced EAE, a mouse model of MS. Treatment was initiated on Day 7 post-immunization. While none of the three doses of 7ND led to a significant improvement in disease score, 7ND-FC inhibited clinical disease during most of the period between Day 15 and Day 32 (Fig. 10). A statistically significant shift in the onset of disease was observed for the 7ND-Fc-treated group with the average onset of disease moving from Day 14 (control) to Day 29 ($P = 0.001$, not shown). Treatment with 7ND did not affect disease onset. We observed anti-human Fc antibodies in the serum of the mice after termination of the study (data not shown). This might be the reason for the limited efficacy at the later time points.

**In vivo depletion of CCR2$^+$ cells by ADCC**

In order to test whether an antibody-dependent cellular cytotoxicity (ADCC)-based mechanism might be responsible for the observed efficacy of 7ND-Fc compared with 7ND alone, we monitored the number of CCR2-positive cells, represented by Ly6C$^+$ and Ly6G$^+$ monocytes and macrophages upon treatment with 7ND-Fc. A rat IgG2b-based anti-CCR2 antibody known to deplete CCR2$^+$ cells in mice (MC-21, Mack et al., 2001) was used as a positive control. While we observed cell depletion for the control antibody, we did not detect ADCC activity for 7ND-Fc as expected for a human Fc administered to mice (Fig. 11).

**Discussion**

The human chemokine CCL2 and its receptor CCR2 have been shown to be implicated in a number of infectious and autoimmune diseases (Charo and Ransohoff, 2006; Proudfoot, 2008), neurological disorders (Ransohoff, 1997; Huang et al., 2000), atherosclerosis (Barlic and Murphy, 2007; Zhao, 2010) and cancer (Yadav et al., 2010). CCR2, a member of the GPCR family and the only high-affinity receptor for CCL2, has therefore been considered an attractive therapeutic target.
Clinical trials aimed at blocking the CCL2–CCR2 axis have been initiated in several indications including RA, MS, atherosclerosis and type II diabetes (Tesch, 2008; Igoillo-Esteve et al., 2010). An alternative strategy to the blockade of CCR2 signaling could be the depletion of CCR2+ cells. Several groups have recently reported the development of agents targeting CCR2-expressing cells, such as a bispecific depleting antibody (Schneider et al., 2005), a GM-CSF-CCL2 fusokine (Rafei et al., 2009) or a fusion protein of CCL2 with the A1 domain of Shigella dysenteriae holotoxin (McIntosh et al., 2009).

7ND, a CCL2 mutant that lacks amino acids 2–8, was shown to be a CCR2 antagonist (Zhang et al., 1994). In contrast, another CCL2 mutant, P8A, is an obligate monomer like 7ND, yet retains full receptor binding affinity as well as chemotaxis and calcium mobilization activity in vitro (Paavola et al., 1998). These data suggest that CCL2 binds and activates its receptor as a monomer, although native CCL2 has been shown to also form dimers and tetramers (Lau et al., 2004). Our original goal was to construct a 7ND-Fc fusion protein in order to overcome the limitations of 7ND that are connected with the short serum half-life of the protein for testing in animal models of disease. The fusion of an immunoglobulin Fc to a protein is an established method to improve the pharmacokinetic properties and simplify protein production and purification (Huang, 2009).

We were able to show that, as expected, the serum half-life significantly increased by fusion of an Fc to 7ND. However, when tested in chemotaxis assays, the 7ND-Fc fusion protein evoked a small but distinct chemotactic response in THP-1 cells, and a robust response in CCR2-transfected L1.2 cells, contrary to the lack of signaling previously reported for 7ND. We hypothesized that this phenomenon is related to the fact that Fc naturally forms a stable homodimer, so a 7ND domain fused to Fc will be presented in a bivalent format. We therefore decided to construct a heterodimeric 7ND-Fc protein displaying only one 7ND moiety, using the technology ‘strand exchange engineered domain’ (SEED) CH3 heterodimers, that has been described recently (Davis et al., 2010). SEED CH3 domains are a complementary pair of chimeras of an IgG and an IgA strand, which preferentially heterodimerize with one another. The resulting 7ND-SEED protein consists of 7ND fused to one SEED Fc sequence, paired with the complementary chimeric IgA/IgG CH3 SEED-domain that does not contain a 7ND fusion (Fig. 1).

In contrast to 7ND-Fc, 7ND-SEED failed to provoke a chemotactic response in THP-1 cells and shows a comparable response to 7ND alone in CCR2-expressing L1.2 cells, suggesting that the response observed for 7ND-Fc is due to the bivalent format of the protein. The gain of agonistic activity also appears to depend on the receptor density of the cells being tested. Modified chemokines which are described as antagonists are often in fact partial agonists, as has been shown for the modified RANTES variants, Met-RANTES and AOP-RANTES (Proudfoot et al., 1999). However, these reagents have been shown to be very useful in dissecting the role of their chemokine receptors in disease models (Proudfoot, 2002). As shown in Fig. 3, the binding affinity of the 7ND-Fc fusion constructs is in the low nM range, whereas the agonist activity is only seen around 100 nM, thereby separating the two effects.

Interestingly, the activity evoked by these constructs appears to be pathway dependent. We showed that while neither 7ND-Fc nor 7ND-SEED induced calcium mobilization, 7ND-Fc but not 7ND-SEED induces ERK-phosphorylation. These results suggest that 7ND-Fc mediates its effect...
through one or both of two non-exclusive possible mechanisms: through increased avidity due to presentation of two 7ND moieties and/or by bringing together two CCR2 receptors. CCL2 has previously been reported to induce functional responses through the dimerization of its receptor (Rodriguez-Frade et al., 1999) and it is therefore possible that bivalency leads to the activation of certain signaling pathways. This possibility is supported by the observation of Bruhl et al. that a monoclonal anti-CCR2 antibody at high concentrations activates basophils by crosslinking CCR2 (Bruhl et al., 2007). Calcium mobilization, on the other hand, is only observed when the fully functional ligand CCL2 is used.

Finally, we studied receptor internalization by these proteins. Surprisingly, 7ND-Fc and 7ND-SEED both induced receptor internalization while we did not observe internalization for 7ND. The 7ND-SEED thus shows properties distinct from 7ND and from 7ND-Fc. The reason why 7ND-SEED but not 7ND induces receptor internalization is unclear as both are monovalent. It might be attributed to binding specificities and/or effector functions found in both Fc and SEED-Fc domains, as some antibodies against chemokine receptors have been shown to mediate internalization (Blanpain et al., 2002).

However, we have shown that 7ND-Fc can be used as a reagent to abrogate CCR2 activity in disease models, as it showed efficacy in MOG-induced EAE, a mouse model of MS. Since 7ND has always previously been tested in disease models by gene therapy, the fact that it was ineffective in this EAE model suggests that constant receptor coverage is necessary, which could not be achieved by administration of a protein with such a short half-life, under a daily dosing regimen. Fusion of 7ND to Fc significantly improved serum half-life, which may explain the enhanced efficacy observed compared with 7ND. In addition, we confirmed that the efficacy observed was unlikely to be due to 7ND-Fc-mediated depletion of CCR2+ cells in vivo through an ADCC-based mechanism, since in a separate in vivo experiment we did not observe a reduction in Ly6G+ and Ly6C-positive cells on treatment with the 7ND-Fc fusion compared with an anti-CCR2 depleting Mab. However, as the human Fc format may not necessarily elicit the engagement of the equivalent murine Fc receptors we cannot rule out other effects mediated by the human Fc portion that contribute to the efficacy in vivo. On the other hand, we did not consider it necessary to perform a second EAE experiment to demonstrate the efficacy of the monovalent 7ND-SEED construct. SEED bodies have previously been shown to have similar half-life to wild-type IgG (Davis et al., 2010) and although SEED bodies have been reported to retain critical Fc effector functions on human cells in vitro, ADCC would still be unlikely to occur in a heterologous system. In addition, in our experience, modified proteins with partial agonist activities are not suitable for further development.

It should be noted that five Fc fusion-based drugs are currently on the market, and many more are in different stages of clinical trials (Huang, 2009). We show here that the fusion of an Fc to a protein, creating a bivalent construct, can significantly change the initial properties of the protein and, as in this case, increase unwanted partial agonist properties of the antagonist protein. Finally, we describe the use of the recently described SEED format which allows the production of a monovalent Fc fusion. The ability to routinely produce heterodimeric Fc fusion proteins presents drug designers with the opportunity to use the favorable properties of Fc while avoiding unwanted effects that may derive from bivalency.

Supplementary data
Supplementary data are available at PEDS online.

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References