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A Novel Recombinant Fusion Protein Encoding a 20-Amino Acid Residue of the Third Extracellular (E3) Domain of CCR2 Neutralizes the Biological Activity of CCL2

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CCL2 is a key CC chemokine that has been implicated in a variety of inflammatory autoimmune diseases and in tumor progression and it is therefore an important target for therapeutic intervention in these diseases. Soluble receptor-based therapy is a known approach for neutralizing the in vivo functions of soluble mediators. Owing to the complexity of seven-transmembrane G protein-coupled receptors, efforts to generate neutralizing soluble chemokine receptors have so far failed. We developed a strategy that is based on the generation of short recombinant proteins encoding different segments of a G protein-coupled receptor, and tested the ability of each of them to bind and neutralize its target chemokine. We show that a fusion protein comprised of as few as 20 aa of the third extracellular (E3) domain of the CCL2 receptor, stabilized by the IgG H chain Fc domain (E3-IgG or BL-2030), selectively binds CCL2 and CCL16 and effectively neutralizes their biological activities. More importantly, E3-IgG (BL-2030) could effectively suppress the in vivo biological activity of CCL2, attenuating ongoing experimental autoimmune encephalomyelitis, as well as the development of human prostate tumor in SCID mice. The Journal of Immunology, 2009, 183: 732–739.

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3 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis.

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Materials and Methods

Construction of soluble receptors

Human and mouse cDNA encoding the constant region (Fc, Hinge-CH2-CH3) IgG1 were constructed as follows: human Fc was generated by RT-PCR on RNA extracted from human PBMC that was cultured for 4 days with LPS and IL-4. The primers used for this reaction were 5′-ctcag CCAAAttTGGGACAAAC and 3′-ggccgg ATTACCCGGGAGCAAGGAGA (AF237583). The mouse Fc was extracted from Con A-stimulated spleen cells and the primers were 5′-ccggcCTGGGAGGCCTCCA and 3′-gaacaa TTTGGGCGGTTTACCAAGA GAGTGGGAGA (L53057).

The PCR products were digested with Xhol and Apat and ligated into mammalian expression/secretion vector pSecTagHyb B2 (Invitrogen Life Technologies). A different set of primers, 5′ (sense) and 5′ (antisense), were used to PCR amplify the cDNA encoding human (28) and mouse (NM000915) (29) domains of CCR2 from the PC-3 and the TRAMP mouse C-1 cell line (30), respectively. The cDNA encoding the human CXCRI4 (NM001008540) (31) was obtained from the PC-3 line. Primers were designed as follows: human CCR2 N terminus 5′-CCCAAG CTTATGTCGGACACTCTGTTCCTGCTGTT 3′-CCGGCCTGGTATCCCACTGGT and mouse CCR2-E1 5′-CCCAAGACT TTCTGCTGCAATGTAATGGGT 3′-CCGGCCTGGTATCCCACTGGT.

The expression and purification of fusion proteins

Expression and purification of the various fusion proteins was done using CHO-dhfr (DG44) cells provided by Dr. L. Chasin (Columbia University, New York, NY) according to the method described in detail (32). The fusion protein was expressed as a disulfide-linked homodimer similar to its mouse counterpart.

CHO dhfr (DG44) cells were used to generate cDNA encoding the different human (NM000647) and mouse (NM001008540) (31) was obtained from the PC-3 line. The cDNA encoding the constant region (Fc, Hinge-CH2-CH3) was extracted from Con A-stimulated spleen cells and the primers were 5′-CCCAAGCTTACTAAATGCCA and 3′-CCGCTCGAGTTTGCACAT (28) and mouse (NM000915) (29) domains of CCR2 from the PC-3 and the TRAMP mouse C-1 cell line (30), respectively. The cDNA encoding the human CXCRI4 (NM001008540) (31) was obtained from the PC-3 line. Primers were designed as follows: human CCR2 N terminus 5′-CCCAAG CTTATGTCGGACACTCTGTTCCTGCTGTT 3′-CCGGCCTGGTATCCCACTGGT and mouse CCR2-E1 5′-CCCAAGACT TTCTGCTGCAATGTAATGGGT 3′-CCGGCCTGGTATCCCACTGGT.

Production of anti-human CCL2 mAb

BALB/c mice were administered 50 µg of human (h)CCL2 in CFA, followed by six injections every 3 wk, of 50 µg of hCCL2 in IFA. Splenocytes were isolated and fused with NS0 myeloma cells by standard procedures (33). Hybridoma supernatants were first screened for their ability to bind hCCL2 by using an ELISA immunoassay. Selected clones were then tested for their binding specificity by Western blotting using different chemokines (PeproTech), and for their in vitro ability to inhibit CCL2-induced migration of the human monocyte cell line THP-1 in a Boyden chamber (Corning; Costar). Our anti-CCL2 mAb selectively binds and neutralizes human CCL2 with low cross-reactivity to its mouse counterpart.

Production of anti-hCCL2 mAb

The evaluation of the affinity of our anti-hCCL2 mAb to CCL2 was conducted by indirect competitive ELISA (34). Briefly, microtiter plates were coated with recombinant CCL2 (dilution 1:10,000, 200 µl/well). After overnight incubation at 4°C, the plates were washed three times with washing buffer, blocked with 1% BSA in PBS (w/v, 250 µl/well), and incubated for 20 min. To titrate mAb affinity, a mixture of 100-2000 ng/100 µl of unlabelled mAbs and 100 ng/100 µl of 125I-labelled mAb was added to each well for 90 min at room temperature. Wells were washed three times and radioactivity was counted in a gamma counter.

Evaluation of the affinity of our anti-hCCL2 mAb to CCL2

The specificity binding of the soluble receptors was detected by an ELISA as follows: each well was coated with 10 ng of either mouse or human proteins, respectively—CCL2, CCL4, CCL5, CCL7, CCL7, CCL8, CCL11, CCL16, CCL19, CCL20, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL1, IL-1β, CD40L (PeproTech) using coating buffer (PBS X1), and incubated at 4°C overnight. Wells were incubated with 200 µl of 1% BSA/PBS blocking buffer for 1 h at room temperature. Soluble chemokine receptors were added (50 µg/ml) in 1% BSA/PBS buffer (50 µl per well) and incubated overnight at 4°C and washed four times with PBS/Tween 20 (0.05%). Then 50 µl of goat anti-human IgG-HRP (Jackson ImmunoResearch) was added at 1/10,000 in 1% BSA/PBS for 1 h and washed four times with PBS/Tween 20 (0.05%). The substrate solution (tetramethylbenzidine) was then added at 50 µl/well. After a blue color appeared, the reaction was terminated by adding 50 µl of H2O2 (1 M). OD was determined at 450 nm with the reference filter set to 620 nm.

Detection of mouse CCL2 was done by ELISA development kit (Mouse CCL2/JE ELISA kit; R&D Systems) and conducted according to the manufacturer’s instruction.

In vitro chemotaxis assays

Chemotaxis assays in which the chemokine-induced migration of mouse cell lines (THP-1 and RAW 264.7 cells, 1 × 10⁶) were determined, were performed in a Transwell system (5-µm pore size, Corning; Costar). Chemotaxis assays in which the chemokine-induced migration of PC-3 cells (1 × 10⁶) were conducted using the Cytoselect cell migration assay (8-µm pore size; Cell Biolabs. Briefly, cells were loaded into the upper chamber of the two systems. The lower chambers were loaded with 20 ng/ml chemokines (h/m CCL2, h/m CCL7, h/m CCL8, h/m CCL11, h/m CCL16, b/c CCL13, mCCL12; PeproTech) and 50 µg/ml soluble receptors (h/m E3-Ig) or Abs. Cells were let to migrate for 2 h under a humidified 7.5% atmosphere at 37°C. The content of the lower chambers was collected and counted using the FACScalibur (BD Biosciences). The chemotaxis index was then calculated by dividing the number of migrating cells in the presence of chemotractant by the number of cells migrated in its absence.

Evaluation of IC₅₀

Chemokine-induced cell migration was assessed using 5-µm pore Transwell filter membranes (Costar). A total of 1 × 10⁶ THP-1, or RAW 264.7 cells, were loaded into each Transwell filter. Soluble receptors (h/m E3-Ig) or mAbs (our anti-hCCL2 mAb and commercial anti-mCCL2 mAb; R&D Systems) were added in the lower chamber at different concentrations (0.01–100 µg/ml) with 20 ng/ml CCL2. Plates were then incubated at 37°C for 2 h. The migrated cells were collected and counted by FACScalibur (BD Biosciences). The IC₅₀ calculation was performed by Origin software (OriginLab).

In vitro proliferation assay

A total of 1 × 10⁶ PC-3 cells were cultured in 96-well flat-bottom tissue culture plates in their respective medium. After 24 h, wells were supplemented with fresh medium, 200 µg/ml neutralizing Abs, 200 µg/ml soluble receptors, or 200 µg/ml total IgG from preimmune mice. Cells were cultured in the presence and absence of these supplements for 5 days. Each well was pulsed with 2 µCi of thymidine (specific activity 10 Ci/mmol) for the final 16 h. The cultures were then harvested on fiberglass filters. Results are shown as the mean cpm of six replicates ± SE from three independent experiments, divided by the mean cpm of control cells.

Animal models

An animal model of EAE used C57BL/6 female mice purchased from Harlan Laboratories and maintained in independent ventilated cages under SPF conditions for at least 5 wk. Mice were then injected with a single dose of either 100 µg of myelin oligodendrocyte glycoprotein (MOG) 35-55 (peptide, Genzyme; Boston, MA) or saline intraperitoneally. For passive transfer, mice were i.v. injected with 0.5 ml of a 1:1 MOG 35-55 emulsion in complete Freud’s adjuvant (CFA) 2 wk before induction of EAE. Animals were sacrificed by cervical dislocation on day 10 after MOG injection for the preparation of spinal cord homogenates or on day 14 for the collection of sera for ELISA. Data was statistically analyzed using nonparametric Mann-Whitney U test or student’s t-test as indicated in the figure legends.
pathogen-free conditions. At 6 wk of age, mice were subjected to active disease induction by a single administration of MOG 33–55 emulsified in CFA as described elsewhere (35). At the onset of disease only mice with an apparent clinical manifestation of disease were selected and separated into equally sick groups for therapy. Animals were then monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, total hind limb paralysis, accompanied by an apparent front limb paralysis; and 4, total hind limb and front limb paralysis.

The animal xenograft model of prostate cancer used SCID/Beige male mice purchased from Harlan Laboratories and maintained in independent ventilated cages under pathogen-free conditions. At 6 wk of age, mice were s.c. injected between the two flanks with $5 \times 10^6$ PC-3 cell line (American Type Culture Collection). Mice were monitored daily for tumor volume. Tumor diameters were measured using a caliper. Tumor volume was calculated using the formula $V = \frac{a \times b^2}{6}$, where $a$ is the longest dimension, and $b$ is the width.

Histology

Spinal cords were subjected to histology analysis. Briefly, tissue samples were fixed overnight in 4% paraformaldehyde in PBS, then dehydrated, paraffin-embedded, and sectioned into 5-μm sections. Sections were then deparaffinized and stained with H&E and with Luxol fast blue.

Statistical analysis

The significant difference was examined using Student’s t test. Values for $p < 0.05$ were considered statistically significant.

Results

A fusion protein encoding 20 aa of the E3 domain selectively binds CCL2 and neutralizes its biological function

In attempt to identify the CCL2 binding site on CCR2, we have cloned the first, second, and third extracellular loops of human CCR2 (E1, E2, and E3, respectively), as well as its N-terminal region and generated four different fusion proteins stabilized by human Ig (Fc) as follows: E1-Ig, E2 of human CCR2 (E2-Ig), E3 of human CCR2 (E3-Ig), the E3-N-terminal region-Ig of human CCR2 (E3+N-Ig), the N-terminal region of human CCR2 (N-Ig), and the N-terminal region-Ig of human CXCR4 (CXCR4-Ig). Results are shown as mean triplicates ± SE.

![FIGURE 1.](image) CCR2 domain E3 binds CCL2 independently of the N-terminal domain of the receptor. A, Schematic presentation of CCR2 domain structure and the related constructs encoding the cloned human CCR2-Ig fusion proteins. B, SDS-PAGE analysis of the different CCR2-Ig fusion proteins shown in a Coomassie blue staining, under reducing and nonreducing conditions: N-terminal, E2, E1, E3, and E3 plus N-terminal under reducing (+2-ME) and nonreducing (−2-ME) conditions. C, Comparative analysis of CCL2 binding to human CCR2-Ig fusion proteins and human CXCXR4-Ig (N-terminal region) as determined by ELISA: E1 of human CCR2 (E1-Ig), E2 of human CCR2 (E2-Ig), E3 of human CCR2 (E3-Ig), the E3-N-terminal region-Ig of human CCR2 (E3+N-Ig), the N-terminal region of human CCR2 (N-Ig), and the N-terminal region-Ig of human CXCR4 (CXCR4-Ig). Results are shown as mean triplicates ± SE.

![FIGURE 2.](image) Human CCR2 E3-Ig selectively binds CCL2 and CCL16 and inhibits their chemokine-induced migration. A, Inhibition of CCL2-induced chemotaxis of THP-1 cells by CCR2-based fusion proteins. The chemotaxis index of THP-1 cells was determined in the absence or presence of 20 ng/ml CCL2 and 50 μg/ml soluble domains. Results are shown as mean triplicates ± SE. B, THP-1 cells were tested for their ability to migrate toward additional natural ligands of CCR2 (20 ng/ml each) in the presence of 50 μg/ml hE3-Ig. Results are shown as mean triplicates ± SE. C, Comparative analysis of E3-Ig binding to different recombinant human chemokines, and to CD40L and IL-1β, in comparison to CCL2 and anti-CCL2 Ab (ELISA). Results are shown as mean triplicates ± SE.
of each of our fusion proteins. Fig. 1C shows that of all fusion proteins the E3 domain, the N-terminal domain, or their fusion (E3-N-terminal) bind CCL2, with the first two exhibiting a slightly higher binding (OD$_{450}$ nm 0.96/0.11006 0.08 compared with 0.74/0.06, $p<0.001$).

Fig. 2A shows the ability of each domain to inhibit the CCL2-induced migration of THP-1 cells. In these recombinant fusion proteins, the E3 domain alone (E3-Ig or BL-2030) not only could significantly inhibit CCL2-induced migration of THP-1 cells (chemotaxis index of 1.32/0.2 compared with 14.34/1.5, $p<0.001$) but could also do so better than each of the other fusion proteins, including the E3-N-terminal fusion (chemotaxis index of 1.32 ± 0.2 vs 5.9 ± 0.6, $p<0.01$). Human CCR2 binds multiple chemokines, including CCL2, CCL8, CCL16, CCL7, CCL11, and CCL13. We have determined the ability of human E3-Ig to inhibit the migration of THP-1 cells induced by each of these chemokines, as well as its direct binding to each of them (ELISA). Our observations clearly show that of these chemokines, E3-Ig (BL-2030) selectively inhibits THP-1-induced migration of two only of them: CCL2 and CCL16. It also exclusively binds both chemokines (Fig. 2C). Its ability to block CCL2-induced migration is significantly higher than to inhibit CCL16-induced chemotaxis under the same conditions (Fig. 2B, $p<0.01$).

**E3-Ig exerts lower affinity to CCL2 in comparison to mAb, yet effectively and selectively suppresses the biological activities of CCL2**

The binding affinity of E3-Ig to recombinant CCL2 was determined using the Biacore technology. As shown in Fig. 3A, E3-Ig exhibited specific binding to CCL2 compared with human Fc, which did not show any binding to CCL2, with a calculated affinity of $2.02 \times 10^{-7}$ M. The binding affinity of our anti-CCL2 mAb, determined as described in Materials and Methods, is $6.5 \times 10^{-9}$ M, which is ~300-fold higher than E3-Ig. We then examined for each of them the IC$_{50}$ required for the inhibition of CCL2-induced migration of THP-1 cells. Fig. 3B shows a dose-dependent inhibition assay for both CCL2 inhibitors, showing that although E3-Ig exhibits low affinity to CCL2, its CCL2 neutralization activity was comparable with the high affinity anti-CCL2 Ab (IC$_{50}$ of 2 μg/ml compared with 2.5 μg/ml, respectively).

**E3-Ig suppresses ongoing EAE in C57BL/6 mice**

We have then cloned the reciprocal E3 domain of murine CCR2, and constructed the murine E3-Ig fusion protein. In murine CCR2 binds CCL2, CCL8, CCL7, CCL11, CCL12, and CCL13 (CCL16 is a human chemokine). The ability of murine E3-Ig to bind and
inhibit chemokine-induced migration of the mouse monocytic cell line (RAW 264.7) in response to each of these chemokines was determined. Our observations clearly show that of these chemokines mE3-Ig exclusively inhibits the CCL2-induced migration of RAW 264.7 cells (Fig. 4 A). It also solely binds this chemokine (Fig. 4 B). We then examined the IC50 required for the inhibition of murine CCL2-induced migration of RAW 264.7, according the protocol used in Fig. 2 B. Our results show that the IC50 of murine E3-Ig (5.2 μg/ml) is comparable to the commercially available control mAb (clone 123616; R&D Systems) (Fig. 4 C), which is 4.7 μg/ml.

Although mice lacking CCL2 displayed markedly reduced EAE (5), there is a dispute whether its targeted neutralization in wild-type rodents suppresses the disease (6, 7, 36). Based on its high specificity in binding and neutralization of CCL2 we have explored the ability of E3-Ig to interfere in the regulation of ongoing EAE in C57BL/6 mice.

These mice were subjected to active induction of EAE and, following the onset of disease (day 15), treated (every 3 days) with 200 μg/mouse of E3-Ig, control mouse IgG1, or PBS and monitored for disease progression by an observer blind to the experimental protocol (Fig. 4 D). We show that administration of E3-Ig (BL-2030) rapidly suppresses EAE (mean maximal score of 1.3 ± 0.2, compared with 3 ± 1.2 and 3.3 ± 0.9 in PBS or control IgG treated mice, respectively, p < 0.01). At the peak of disease, representative mice were sacrificed, and lumbar spinal cords were subjected to histological analysis. Fig. 4 D shows the results of representative sections (of 18 sections per group), showing a marked decrease in leukocyte infiltrate around HEV of mice treated with E3-Ig but not with control IgG or PBS. A complementary Luxol fast blue staining was conducted to verify that recovery is associated with reduction in demyelination that occurs during EAE in C57BL/6 mice (Fig. 4 E). Thus, E3-Ig (BL-2030) effectively suppresses an ongoing EAE disease. Finally, We have
measured the levels of CCL2 in circulating blood 1 day after the second mE3-Ig administration during EAE (Fig. 4D). We identified an 1.8-fold increase in CCL2 levels in the circulation of treated mice (57.15 ± 6.1 ng/ml in E3-Ig-treated mice compared with 30.1 ± 4.2 ng/ml in PBS-treated mice).

E3-Ig inhibits the proliferation and CCL-2 induced migration of PC-3 cells in vitro

Three human prostate cancer cell lines are commonly used in xenograft experiments: LNCaP, an androgen-dependent cell line derived from a patient’s lymph node, and DU-145 and PC-3, both androgen-independent cell lines, derived from brain and bone metastases, respectively. Of these lines, PC-3 is the most aggressive (37) and has therefore been selected for our in vivo xenograft studies. After verification of CCR2 expression (FACS analysis, data not shown), we tested whether hE3-Ig would affect CCL2-induced migration of PC-3 cells in vitro (Fig. 5A). We show that hE3-Ig effectively blocks CCL2-induced chemotaxis of PC-3 cells (chemotaxis index of 3.16 ± 0.2 vs 10.3 ± 0.09, p < 0.01), and that the migration of these cells is directed via the CCL2-CCR2 interaction (Fig. 5A). An anti-CCR2 mAb (38) provided by Dr. C. Martinez-A (Centro Nacional de Biotecnologia, Universidad Autonoma de Madrid, Madrid, Spain) also effectively suppressed CCL2-induced migration of these cells (chemotaxis index of 2.6 ± 0.2 vs 10.3 ± 1.4, p < 0.01) (Fig. 5A). We then determined the ability of this soluble receptor to inhibit the proliferation/growth of PC-3 cells. As shown in Fig. 5B, addition of E3-Ig to cultured PC-3 cells significantly reduced the proliferation of these cells (41 ± 2.4 × 10^3 cpm vs 100 ± 8.7 × 10^3 cpm, p < 0.01). Similar results were obtained using the anti-CCR2 mAb (28 ± 1.9 × 10^3 cpm vs 100 ± 8.5 × 10^3 cpm, p < 0.01).
E3-Ig inhibits tumor growth in a prostate cancer xenograft model

Next, we explored the ability of hE3-Ig to suppress the growth of PC-3 in SCID/Beige mice. Briefly, mice were implanted with $5 \times 10^6$ PC-3 cells and beginning on day 7, when tumors were clearly identified in all animals, were treated (twice a week) with PBS, isotype-matched IgG1, or 50, 100, or 200 μg/mouse of hE3-Ig and monitored for tumor growth (Fig. 6). Our results clearly show a dose-dependent blockade of primary tumor growth in E3-Ig-treated mice (tumor size at day 40, 1550 mm$^3$ ± 123.7 and 2020 mm$^3$ ± 134.8 in control groups, compared with 90.5 mm$^3$ ± 26.1 and 505 mm$^3$ ± 91.1 in those treated with 200 and 100 μg of E3-Ig, respectively, $p < 0.001$). Most importantly, E3-Ig inhibitory effect was still observed 15–35 days following treatment termination, hinting to its therapeutic potential as anticancer agent.

After determining the dissociation constant ($K_d$) and neutralizing activity ($IC_{50}$) of our anti-human CCL2 mAb in comparison to hE3-Ig (Fig. 3), we have compared their ability to suppress the growth of human prostate cancer cell line (PC-3) in SCID mice. It has been previously shown that in this xenograft model even though both the murine and tumor-derived CCL2 contribute to tumor development, targeted neutralization of the human-derived chemokine markedly suppresses tumor development (14). Our results clearly show that under the same experimental conditions, both equally very effectively suppressed tumor development (Fig. 6B) despite the major difference in anti-human CCL2 mAb in comparison to hE3-Ig binding affinity to CCL2 (~300-fold higher for the Ab).

Discussion

In this study, we describe a novel recombinant soluble form of the CCL2 receptor, E3-Ig (BL-2030). We have shown that E3-Ig selectively binds CCL2 and neutralizes its biological activity, mainly the chemotaxis of human monocytes in vitro. In addition, it inhibits the proliferation and migration of the human prostate cancer cell line PC-3 that expresses CCL2 and its receptor. The in vivo activity of E3-Ig was demonstrated in two separate disease models in which the CCL2-CCR2 pathway was shown to play a role, the EAE model of MS and the PC-3 prostate cancer model. E3-Ig (BL-2030) significantly suppressed ongoing EAE disease and exhibited dose-dependent inhibition of PC-3 tumor growth. The inhibition of PC-3 cell proliferation and migration in vitro together with the suppression of tumor growth in vivo indicate an anti-tumorigenic and anti-metastatic potential of E3-Ig. In support of our results, a previous study has demonstrated that an anti-CCL2-neutralizing Ab attenuates tumor burden in the PC-3 prostate cancer model (14).

Chemokine receptors belong to the superfamily of seven-transmembrane G protein-coupled receptors (2) that span the plasma membrane seven times generating three loops (domains). Their three-dimensional structure is dependent on plasma-membrane stabilization, and therefore the generation of soluble chemokine receptors that are highly effective in neutralizing their target chemokines is still a major challenge. In an attempt to overcome this obstacle, we generated short recombinant proteins encoding different segments of the G protein-coupled receptor CCR2, and tested their ability to bind and neutralize its target chemokine CCL2. We show that although both the E3 domain and the N terminus of CCR2 bind CCL2, only the E3 domain alone, or in combination with the N-terminal region, neutralizes the basic biological activity of CCL2; chemotactraction of monocytes. Surprisingly, the E3 domain alone comprising only 20 aa of CCR2 (fused to Ig) was the most effective inhibitor of CCL2-induced chemotaxis in vitro.

Neutralizing mAbs and soluble receptor-based fusion proteins were shown to be effective in the treatment of autoimmune diseases and cancer. Use of anti-TNF-α mAbs and recombinant soluble TNF-α receptor fusion proteins is a classical example of successful treatment of rheumatoid arthritis and other related autoimmune diseases (39–41). One of the major disadvantages of mAb-based therapies is their tendency to elicit anti-idiotypic neutralizing Abs, as a part of a natural regulatory network (42). From this perspective, soluble receptors have a therapeutic advantage. The CCL2/CCR2 axis has been implicated in the pathophysiology of a wide range of both acute and chronic inflammatory conditions, such as rheumatoid arthritis, MS, atherosclerosis, uveitis, asthma, psoriasis, diabetes, inflammatory bowel disease, lupus nephritis, transplant rejection, and several CCL2/CCR2 antagonists are currently under clinical development.

The role of CCL2 in EAE has been studied by either using deficient mice, or by administering anti-CCL2 polyclonal Abs to EAE in mice or rats. Izikson et al. (43) showed that mice lacking CCR2 are EAE-resistant. Nevertheless, at least six different chemokines bind CCR2, therefore other chemokines, aside of CCL2, might contribute to EAE resistance in CCR2-deficient mice as demonstrated in that study. A complementary publication of Huang et al. (5) showing that mice lacking CCL2 display a markedly reduced form of disease, further emphasizes the pivotal role of CCL2 in the pathogenesis of EAE. Thus far the evidence that targeting CCL2 alone, in wild-type rodents suppresses EAE emerged from studies that used polyclonal Ab-based therapies, with their limitations. There is a dispute in this studies whether targeted neutralization of CCL2 in wild-type rodents suppresses the disease (6, 7, 36). We show in this study that in C57BL/6 EAE mice injection of E3-Ig, that specifically and exclusively binds and neutralizes CCL2 (Fig. 4, A–C), effectively suppressed an ongoing disease (Fig. 4, D and E) further implies that this chemokine plays a pivotal role in EAE, as previously demonstrated in mice lacking CCL2 (5).

It has been reported that administration of anti-CCL2 mAbs to patients suffering from rheumatoid arthritis led to a 2000-fold increase in their circulating CCL2 (44), which may explain, in part, why therapy was ineffective. In the current study we show that repeated administration of E3-Ig only moderately increased circulating blood levels CCL2 (1.8-fold increase). It is possible that in human therapy with E3-Ig would also result in a moderate increase in circulating CCL2. This effect would be a major advantage to the recipients, though this feature has yet to be carefully detected along clinical trails.

In addition of being a key regulator of monocytes infiltration to sites of inflammation, CCL2 was shown to possess protumorigenic and proangiogenic functions. The mechanisms by which CCL2 promotes tumor progression are still unclear. CCL2 is primarily responsible for the recruitment of tumor infiltrating macrophages into the tumor site, stimulating angiogenesis and metastasis. In addition, CCL2 has been shown to have direct effects on the tumor cells in several neoplasms including breast, lung, cervix, ovary, sarcoma, and prostate, inducing cancer cell proliferation, migration, and survival.

We clearly show that E3-Ig well suppresses the establishment of human tumor cells in SCID mice. Our human E3-Ig (BL-2030) binds and neutralizes both CCL2 and CCL16 (Fig. 2). Human cancer cells produce both chemokines (9). CCL16 is also likely to contribute to tumor invasion and angiogenesis (45). Therefore it could be that its neutralization by BL-2030 also contributes to tumor suppression in a xenograft model of cancer (Fig. 6).
E3-Ig (BL-2010) represents a novel approach for inhibiting CCL2. Its potent anti-tumorigenic effect, together with its anti-inflammatory activity, suggests a therapeutic potential for the treatment of autoimmune diseases, as well as various malignancies. Further studies exploring the therapeutic potential of E3-Ig in additional inflammatory disease models and various cancer models are required.

Disclosures

N.K., L.I., G.W. and Y.Z. hold a pending patent on therapy of inflammatory activity, suggests a therapeutic potential for the treatment of autoimmunity and cancer using CCL2 E3-Ig that has been licensed out to BiolineRx. L.K., M.A.S., and A.E. are research scientists at BiolineRx and preformed part of the in vitro analysis within the manuscripts and also participate in discussing the results.

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