Functional Defect of Peripheral Neutrophils in Mice With Induced Deletion of CXCR2

LiPing Liu,† MeiZhang Li,† Lisa C. Spangler, Charles Spear, Mike Veenstra, Lindsey Darnall, Cathleen Chang, Anne C. Cotleur, and Richard M. Ransohoff*

Department of Neuroscience, Neuroinflammation Research Center, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio

Received 2 January 2013; Revised 15 March 2013; Accepted 26 April 2013

Summary: Type 2 CXC chemokine receptor CXCR2 plays roles in development, tumorigenesis, and inflammation. CXCR2 also promotes demyelination and decreases remyelination by actions toward hematopoietic cells and nonhematopoietic cells. Germline CXCR2 deficient (Cxcr2−/−) mice reported in 1994 revealed the complexity of CXCR2 function and its differential expression in varied cell-types. Here, we describe Cxcr2fl/fl mice for which the targeting construct was generated by recombineering based on homologous recombination in E. coli. Without recombination Cxcr2fl/fl mice have CXCR2 expression on neutrophils in peripheral blood, bone marrow and spleen. Cxcr2fl/fl mice were crossed to Mx-Cre mice in which Cre recombinase is induced by Type I interferons, elicited by injection with polyinosinic-polycytidylic acid (poly(I:C)). CXCR2-deficient neutrophils were observed in poly(I:C) treated Cxcr2fl/fl::Mx-Cre− (Cxcr2-CKO) mice, but not in poly(I:C) treated Cxcr2fl/fl::Mx-Cre− mice. CXCR2 deletion was mainly observed peripherally but not in the CNS. Cxcr2-CKO mice showed impaired neutrophil migration in sterile peritonitis. Cxcr2-CKO mice reported here will provide a genetic reagent to dissect roles of CXCR2 in the neutrophil granulocyte lineage. Furthermore Cxcr2fl/fl mice will provide useful genetic models to evaluate CXCR2 function in varied cell populations.

Key words: CXCR2; chemokine; chemokine receptor; conditional KO mice; neutrophil

INTRODUCTION

CXCR2 was cloned in 1991 (Holmes et al., 1991; Murphy and Tiffany 1991) and is expressed on myeloid cells in the periphery as well as on oligodendrocyte progenitor cells (OPCs) in the central nervous system (CNS). With its seven differentially regulated ligands, CXCR2 shows multiple additional functions beyond chemoattraction for myeloid cell trafficking (Cacalano et al., 1994). On OPCs in the developing rodent spinal cord, CXCR2 interacts with CXCL1, arresting migrating OPCs during development, and promoting the OPC proliferative response to PDGFA (Tsai et al., 2002). CXCR2 also plays a role in wound healing (Devalaraja et al., 2000), acetaminophen hepatotoxicity (Hu and Colletti 2010), bone mineralization, intramembranous bone formation (Bischoff et al., 2011), spontaneous tumorigenesis (Jamieson et al., 2012), cancer metastasis and chemoresistance (Acharyya et al., 2012). Recently we and others found that CXCR2 function on neutrophils plays a role in both autoimmune and toxic demyelination (Carlson et al., 2008; Liu et al., 2010a) as well as myelin repair (Liu et al., 2010b). Importantly, CXCR2 plays orthologous roles in humans and rodents (Mihara et al., 2005).

CXCR2 deficient mice (Cxcr2−/−) (Cacalano et al., 1994) are fragile and infertile, which complicates

1LiPing Liu and MeiZhang Li contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Richard M. Ransohoff, Neuroinflammation Research Center, Department of Neuroscience, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA 44195. E-mail: ransohr@ccf.org

Contract grant sponsor: National Institutes of Health Grants, Contract grant numbers: R01NS032151; NS051400.

Current address for MeiZhang Li: Laboratory of Biochemistry and Molecular Biology, School of Life Sciences, Yunnan University, Kunming 650091, People’s Republic of China.

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/dvg.22401
breeding and disease modeling. We generated Cxcr2fl mice to extend research into cell-type specific and inducible deletion of this pleiotropic receptor. In transgenic mice expressing inducible Cre recombinase under control of the Mx1 promoter (Mx-Cre) (Kuhn et al., 1995), our induction protocol efficiently deleted the floxed Cxcr2 gene in hematopoietic cells. Cxcr2-CKO mice 4 weeks after poly(I:C) injections showed deletion of CXCR2 on peripheral neutrophils and deficient neutrophil migration. Our data indicate that Cxcr2fl mice provide a useful reagent to advance current research into CXCR2 and its chemokine ligands in inflammation, cancer and neurological disease.

RESULTS AND DISCUSSION

Generation of Cxcr2 Conditional Knockout (Cxcr2-CKO) Mice

A Cxcr2 conditional targeting construct was generated by recombineering and electroporated into C57BL/6 ES cells, which were screened by Southern blotting (Fig. 1A). Twenty-five candidate ES cell lines were verified both through 3' screening (Fig. 1A, data not shown) and sequence analysis through the loxP and FRT sites (data not shown). One correct ES clone (clone 1) from seven candidates was re-confirmed by sequence analysis of the loxP and FRT sites (data not shown).

Chimeric founder mice harboring the Cxcr2fl allele were intercrossed to generate Cxcr2fl fl mice. To create the Cxcr2-CKO mouse line, Cxcr2fl fl mice were bred to Mx-Cre mice (Kuhn et al., 1995) and intercrossed to generate Cxcr2fl fl::Mx-Cre + and Cxcr2fl fl::Mx-Cre + genotypes.

Expression of CXCR2 in Cxcr2fl fl Mice

The Cxcr2fl allele was detected with F5FRT and R6FRT primers (Fig. 1B-a). CXC2 was detected by flow cytometry on Ly6G+ neutrophils of Cxcr2fl fl mice, at similar frequency and mean fluorescence

FIG. 1. Generation of a conditional Cxcr2 allele and genotype determination of conditional knockouts by PCR. (A) The strategy for Cxcr2fl mouse generation. (a) Schematic diagram of the Cxcr2 wild-type genomic locus demonstrating a classic two-exon gene structure that is indicated by two blank boxes. In order to screen for the targeted Cxcr2fl mutant allele, both 5' and 3' Southern blot probes (filled boxes) were designed in the genomic regions inside of two homologous arms (HA). Restriction enzymes are in the abbreviated form: Ec, EcoRV, and Ba, BamHI. (b) A Cxcr2fl targeting vector was constructed by recombineering. The targeting region includes a 2-kb 5' HA, a LoxP site that flanks Cxcr2 exon2, a Frt-PGKNeo-Frt cassette that is downstream of Cxcr2 exon2 flanked by another LoxP site and a 3-kb 3' HA. (c) Schematic demonstration of the targeted Cxcr2fl allele. In Southern blot screening, an 8.8 kb-BamHI targeted band (5' probe) and an 11kb- EcoRV targeted band (3' probe) are expected. (d) Schematic diagram depicting the conditional deletion of the Cxcr2 gene through Cre-mediated recombination. (e) Seven targeted embryonic stem cells were screened by Southern blot. 7.1 kb wild-type and 8.8 kb targeted BamHI bands were detected by the 5' Southern probe. 24 kb wild-type and 11 kb mutant-type EcoRV bands are detected by the 3' Southern probe. An 8.8 kb targeted BamHI band is detected by using the PGK-neo probe. Targeting vector was used as the control. (B) Genotype determination of Cxcr2-CKO mice by PCR. (a) Strategy for designing PCR primers to detect the WT locus, the flox allele and the deletion of the flox allele. The primers are shown as arrows. (b) PCR amplification of the genomic DNA using primers for the flox gene before injection of poly(I:C); the higher band (~650 bp) indicates the wild-type allele and the lower band (~450 bp) indicates the flox allele (top). PCR amplification of the genomic DNA using primers to detect the deletion of the flox allele (~400 bp) after poly(I:C) induced recombination (bottom). (c) PCR amplification of the genomic DNA using primers to detect the Mx-Cre transgenes (top). The details for designing the primers specific for Mx-Cre are described in Supporting Information data 1. The bottom figure shows representative PCR results of GAPDH for quality control of the genomic DNA used for (b) and (c).
intensity to \( \text{Cxcr}^2^{+/+} \) mice (Fig. 2). Spleen and bone marrow from \( \text{Cxcr}^2^{β/β} \) and \( \text{Cxcr}^2^{+/+} \) mice showed similar CXCR2 expression patterns as peripheral blood (Supporting Information 2, data not shown).

**CXCR2 Is Stably Deleted by poly(I:C) in Mx-Cre::Cxcr2 CKO Mice**

Intraperitoneal poly(I:C) injections induced Cre recombinase in \( \text{Cxcr}^2^{β/β} \cdot \text{Mx-Cre}^+ \) and \( \text{Cxcr}^2^{β/β} \cdot \text{Mx-Cre}^+ \) mice. Analysis of genomic DNA after poly(I:C) injection showed the \( \text{Cxcr}^2 \) deletion product in samples from \( \text{Cxcr}^2^{β/β} \cdot \text{Mx-Cre}^+ \) and \( \text{Cxcr}^2^{β/β} \cdot \text{Mx-Cre}^+ \) but not \( \text{Cxcr}^2^{β/β} \cdot \text{Mx-Cre}^+ \) or \( \text{Cxcr}^2^{β/β} \) mice (Fig. 1B).

We analyzed CXCR2 protein product on blood leukocytes weekly after poly(I:C) induction. In Cxcr2-CKO mice, CXCR2 deletion on blood neutrophils was time-dependent. To our surprise, deletion of CXCR2 was first observed at 3 weeks post injection (pi) (Fig. 3b). At 4 weeks pi, most Cxcr2-CKO mice showed >90% of blood neutrophils were CXCR2-negative (calculated by the ratio of CXCR2 negative cells in total Ly6G+ cells) (Fig. 3b, Supporting Information 2). Cells from the spleen and bone marrow showed equivalent CXCR2 deletion in the neutrophil lineage (Supporting Information 2). Monitoring until 18 weeks pi, showed stable deletion of CXCR2 (Fig. 3b). Cxcr2-CKO mice showed considerable delay in the appearance of CXCR2-negative circulating neutrophils, as compared with previous studies targeting other cell surface molecules where target-deficient cells are detected within a few days (Tiedt et al., 2008; Ulyanova et al., 2007; Yan, 2008). Before appearance of CXCR2-deficient leukocytes in the circulation, necessary events include activating the Mx1 promoter with poly(I:C)-induced type I IFN, producing Cre recombinase, and recombinating the floxed gene. Thereafter, turn-over of the targeted protein or cells expressing that protein must occur before target-negative cells predominate (Nagy, 2000). It remains plausible that retention of CXCR2-deficient neutrophil progenitors in bone marrow (Köhler et al., 2011) accounts for the failure of CXCR2-negative neutrophils to accumulate in the bloodstream. In particular, neutrophil progenitors remaining CXCR2+ will be privileged for bone marrow exit and that population must be exhausted before CXCR2-deficient cells will appear in the periphery.

\( \text{Cxcr}^2^{β/β} \cdot \text{Mx-Cre}^+ \) mice were born at expected Mendelian ratios and showed normal weight, behavior, fertility and life span. Cxcr2-CKO mice lost weight compared with littermates after efficient CXCR2 deletion on neutrophils (data not shown), reminiscent of
the failure-to-thrive phenotype in germline $\text{Cxcr}^{2/-}$ mice.

**CXCR2-Deficient Neutrophils Show Defective Ligand Scavenging**

Signaling chemokine receptors such as CXCR2 scavenge their ligands (Cardona et al., 2008). CXCR2 ligand CXCL1 was undetectable in plasma of $\text{Cxcr}^{2+/+}$ or $\text{Cxcr}^{2/-/+}$ mice, while being present at high levels in plasma from positive control $\text{Cxcr}^{2/-+}$ animals showing that the $\text{Cxcr}^{2/+/+}$ targeted allele scavenged efficiently (Fig. 4, data not shown). $\text{Cxcr}^{2-/-}$ mice with more than 90% CXCR2 deletion on neutrophils showed dramatically increased plasma CXCL1 in the serum comparable with those in $\text{Cxcr}^{2/-+}$ mice (Fig. 4). However, $\text{Cxcr}^{2-/-}$ mice with less than 90% CXCR2 deletion on neutrophils showed comparable levels of plasma CXCL1 to control mice (Fig. 4). These results indicate that near-complete induction of CXCR2 deficiency on peripheral neutrophils is required to abrogate the scavenging of CXCL1.

**Dose-Dependent Recombination Induced by poly(I:C) in Periphery But Not CNS of $\text{Cxcr}^{2-/-}$ Mice**

After four injections at 2.5 mg/kg poly(I:C), we observed 30% deletion of CXCR2 on neutrophils while 15 mg/kg poly(I:C) induced >90% deletion on neutrophils at 4 weeks pi, 6 weeks pi and 8 weeks pi (Fig. 5a). Concerned with effects of the 15 mg/kg poly(I:C), including inflammatory cytokine (TNF-α, IL-6, or IFNγ) production and sickness behavior

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**FIG. 2.** Expression of CXCR2 in $\text{Cxcr}^{2-/-}$ mice. Peripheral blood cells from $\text{Cxcr}^{2+/+}$ and $\text{Cxcr}^{2/-+}$ mice (bottom) stained with Ly6G and CXCR2 antibodies were analyzed by flow cytometry. Cells from $\text{Cxcr}^{2/-+}$ and $\text{Cxcr}^{2/-+}$ mice (top) were used as positive and negative controls respectively. These data represent at least three independent experiments.
which could confound data analysis for neuroinflammatory disease models, we examined the timing of poly(I:C) injections at lower doses of poly(I:C). At 5 mg/kg poly(I:C) with assay 4 wks pi, we observed partial CXCR2 deletion from circulating neutrophils of mice injected at ages >8 weeks. Using the same dose and timing of assay, we observed >90% CXCR2 deletion on neutrophils of most mice injected at 4 weeks of age (Fig. 5b). Compared with 5 mg/kg, high-doses (15 mg/kg) of poly(I:C) once every other day for four injections did not alter the extent or kinetics for generating CXCR2-negative blood neutrophils in young mice (~3–4 week old). However, high-dose poly(I:C) caused more-efficient CXCR2 deletion on neutrophils in older mice (>8 week old). We concluded that, 5 mg/kg was an appropriate dose of poly(I:C) for the CXCR2 deletion in young Cxcr2-CKO mice avoiding the neurotoxic effects caused by high-dose poly(I:C).

To determine whether the deletion of CXCR2 differed in the periphery from the CNS, we performed qPCR to examine Cre expression. Cre recombinase induction in spleen (200-fold) was far greater than that in the brain (six-fold) (Fig. 5c). To determine if low-level induction of Cre recombinase mediated recombination in CNS parenchyma, we crossed Mx-Cre mice to reporter ROSA26mTmG mice (Muzumdar et al., 2007), and monitored recombination as conversion from tomato red to GFP labeled cells in the CNS. There were no green neuroepithelial parenchymal CD45 negative cells in the CNS 4 weeks after injection of 5 mg/kg poly(I:C) by flow cytometry (data not shown), which is consistent with a previous finding (Kuhn et al., 1995).
Decreased Migration of CXCR2-Deficient Neutrophils in Sterile Peritonitis

To assess neutrophil migration toward an inflammatory stimulus in Cxcr2-CKO mice, the sterile irritant thiglycollate (TG) was administered i.p. Total peritoneal cell number was reduced by about 50% in Cxcr2-CKO mice (0.32 ± 0.02 × 10^6/mL) as compared with the Cxcr2^fl/fl::Mx-Cre^1 group (0.59 ± 0.10 × 10^6/mL, P = 0.022) due to the virtual absence of recruited neutrophils in Cxcr2-CKO mice (Fig. 6a,b). Giemsa-stained cytospin preparations from the peritoneal cavity showed more macrophage-like cells in Cxcr2-CKO mice than in Cxcr2^fl/fl::Mx-Cre^1 mice (Fig. 6c). In Cxcr2^fl/fl::Mx-Cre^1 mice, CXCR2 expression was downregulated on infiltrated peritoneal neutrophils (Fig. 6d) suggesting receptor engagement during migration.

Our current data indicate that the floxed Cxcr2 gene was deleted by induction of Cre recombinase in Cxcr2^fl/fl::Mx-Cre^1 mice, providing opportunities to elucidate the functions of CXCR2 and its many chemokine ligands in murine models of human disorders such as neurodegenerative disease and cancer.

METHODS

Mice

The Cxcr2 targeting construct for generation of a conditional Cxcr2 allele was built by recombineering (Liu et al.).
et al., 2003), based on homologous recombination in *E. coli* and applied to modifying BACs. The Cxcr2 targeting construct DNA was electroporated into C57BL/6 cells. Twenty-five positive candidates from the total 192 ES clones were screened and verified by both Southern hybridization and genomic DNA sequencing. One targeted ES clone was injected into 129SvEv blastocysts to create chimeric mice. Chimeric founder mice (C57BL/6×129/SvEv) were backcrossed with C57BL/6 mice. Germline transmission of the Cxcr2floxed (Cxcr2fl) allele was confirmed by Southern hybridization and PCR genotyping. To establish the Cxcr2-CKO mouse line, Cxcr2fl mice were further bred to Mx-Cre mice (Stock Number: 003556; Strain name: B6.Cg-Tg (Mx1-Cre)1Cgn/J from the Jackson Laboratory). Conditional knockout mice include the Cxcr2-CKO mice. Control mice used for conditional deletion studies included Cxcr2fl/−::Mx-Cre− or Cxcr2fl/− genotypes. ROSA26mTmG mice were obtained from The Jackson Laboratory. All mouse studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic (Cleveland, OH).

**Genotyping**

The Cxcr2fl allele was detected with primers which consist of forward primer F5FRT (AGGGAAATGGGGATATTTGG) and reverse primer R6FRT (GCTTGGCTGGACGTAAACTC). Mice were genotyped by amplification of genomic DNA obtained by tail biopsy as shown by Figure 1b. The Cxcr2fl allele was detected with primers which consist of forward primer F5FRT (AGGGAAATGGGGATATTTGG) and reverse primer R3LoxP2 (CGTCTGTGCCTTCTAAGCCT). This PCR reaction yields a 450bp fragment from the Cxcr2fl allele and 640bp fragment from the Cxcr2fl allele. The presence of the deleted allele was detected with LoxPF3 primer (CTACTAGCATGTTTGAGCCCT) and FrtR primer (CTTGAATAGGGTTGGTGT), the amplified fragment measured 400bp (Fig. 1b). This band was purified with

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FIG. 6. Decreased migration of CXCR2-deficient neutrophils in sterile peritonitis. Cxcr2-CKO and Cxcr2fl/−::Mx-Cre− mice 4 weeks after poly(I:C) injection were injected with 4% aged TG 2 h before analysis. (a) Total cells were collected from the peritoneum and counted on a hemocytometer. (b) Total infiltrated neutrophils in peritoneum were calculated by total cells collected from the peritoneum times the percentage of neutrophils in total cells determined by the staining of peritoneal cells with Ly6G and CD45 antibodies (data not shown). (c) Wright-Giemsa staining of peritoneal cells collected by cytopsin showed neutrophils with multiple lobulated nuclei in the peritoneal exudate of Cxcr2fl/−::Mx-Cre− mice (top) and mononuclear and kidney-shaped nuclei consistent with monocytes in the peritoneum of Cxcr2-CKO mice (bottom). (d) Peritoneal exudate cells and blood cells from Cxcr2fl/−::Mx-Cre− mice were collected and stained with Ly6G and CXCR2 antibodies, then analyzed by flow cytometry. These data represent two independent experiments. Each experiment included three mice per group. **P < 0.01; *P < 0.05.
Injection of poly(I:C) and Analysis of CXCR2 Deletion

Totally, 4- or 8-week-old mice were treated with different doses of poly(I:C) (P1530, Sigma, St. Louis, MO) (2.5 mg/kg, 5 mg/kg, 15 mg/kg) as indicated every other day for a total of four injections. The time after poly(I:C) injection was counted from the last day of injection (Day 0) as days post injection (pi.).

Deletion of CXCR2 from freshly isolated peripheral leukocytes was determined by flow cytometry. Blood was drawn before the first poly(I:C) injection (time point 0) and once per week for the indicated time. Red blood cells were lysed with red cell lysis buffer as described previously (Liu et al., 2010a). For the analysis of percentage of deleted CXCR2 on neutrophils, the peripheral leukocytes were stained with antibody mixtures of CD45APC (Clone: 30-F11; Biolegend), CXCR2PE (Clone: 2A2216; R&D) and Ly6GFITC (Clone: 1A8; Biolegend) and analyzed by flow cytometry. Total cell counts were determined by flow cytometry. Total cell counts were performed by staining with antibodies as indicated in data and analyzed by flow cytometry. Some of the cells from the peritoneum were cyotspun for Wright-Giemsa staining according to the manufacturer’s protocol.

Statistical Analysis

Data are expressed as mean ± SD. Multiple comparisons were statistically evaluated by 1-way ANOVA using Prism 4 (GraphPad Software). The Students’-t test was used for the comparisons of cytokine content, the percentage of CXCR2 deletion. A P value <0.05 was considered as significant. *P < 0.05, **P < 0.01.

ACKNOWLEDGMENTS

The authors thank the Murine Molecular Constructs Laboratory from the Mouse Biology Program at UC Davis and the Case Transgenic and Targeting Facility at Case Western Reserve University. They thank Dr. Qi Shi (Neuroscience Department, Cleveland Clinic) for help with genotyping the mice; they thank the LRI-Flow Core for providing excellent service. Thank you also to Dr. Christie L. White (Molecular Genetics Department) for providing primers and techniques for checking the activation of interferons.

LITERATURE CITED


