Elucidation of Distinct Roles of Guinea Pig CXCR1 and CXCR2 in Neutrophil Migration by Inhibitory Antibodies

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Elucidation of Distinct Roles of Guinea Pig CXCR1 and CXCR2 in Neutrophil Migration by Inhibitory Antibodies

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Abstracts

CXCR1 and CXCR2 are seven-transmembrane G-protein-coupled receptors that are expressed on neutrophils, which mediate the migration of these cells to sites of inflammation in response to chemokine ligands. These receptors are suggested to play different physiological roles because they have different ligand selectivity. IL-8, which is a major chemokine ligand that induces neutrophil migration, is known to be a shared ligand of both CXCR1 and CXCR2, while growth-regulated oncogene α/β/γ, ENA-78, and NAP-2 are selective ligands of CXCR2 in humans. The use of an animal model is necessary to elucidate the distinct functions of CXCR1 and CXCR2 under physiological conditions, and guinea pigs are considered to be an appropriate species for this because of their possession of an IL-8 ortholog and its functional similarity to that in humans. Inhibitors are also necessary to elucidate distinct function, but specific inhibitors against guinea pig CXCR1 and CXCR2 have not been identified. In the work described in this thesis, inhibitory antibodies against these receptors were generated and used to elucidate the distinct roles of guinea pig CXCR1 and CXCR2 in neutrophil migration.

In chapter 1, the background and aim of this research, and the structure of this thesis are introduced. Next, chapter 2 describes the generation of monoclonal antibodies against guinea pig CXCR1 and CXCR2, which specifically inhibit their function.

Because CXCR1 and CXCR2 are multi-transmembrane proteins and the production of these proteins as immunogens was difficult, DNA immunization methods that can induce antibody by *in vivo* expression of immunogens in native conformation were examined. Intramuscular injection followed by electroporation was selected as a result of comparison of DNA immunization methods, and monoclonal antibodies that specifically bound to guinea pig CXCR1 and CXCR2 were generated. To assess the inhibitory activities of these antibodies, CHO-K1 cells stably expressing either guinea pig CXCR1 or CXCR2 were established. CHO-K1 expressing CXCR1 showed migration in response to IL-8, and CHO-K1 expressing CXCR2 showed migration in response to both IL-8 and growth-regulated oncogene α. These results suggest that the ligand selectivity of guinea pig CXCR1 and CXCR2 is consistent with that in humans. In this migration system, the inhibitory activities of the anti-gpCXCR1 and anti-gpCXCR2 monoclonal antibodies against cell migration were observed in a concentration-dependent manner. Taking the obtained findings together, inhibitory antibodies specific to gpCXCR1 and gpCXCR2 were successfully obtained.

As described in chapter 3, the distinct functions of CXCR1 and CXCR2 on guinea pig neutrophils were also elucidated using these inhibitory antibodies. To characterize the guinea pig neutrophils, CXCR1 and CXCR2 protein expression was confirmed on

them using the antibodies, and guinea pig IL-8 and growth-regulated oncogene α were proved to induce dose-dependent migration of the neutrophils. In this migration system, the inhibitory antibodies against CXCR1 and CXCR2 revealed that both CXCR1 and CXCR2 mediate the migration induced by IL-8, while CXCR2 mediates the migration induced by growth-regulated oncogene α in guinea pigs. These results indicate that both CXCR1 and CXCR2 function on the neutrophils of guinea pigs in response to their ligands, similarly to how they do in humans.

In chapter 4, the conclusions of this thesis and future prospects are addressed. In this work, the specific antibodies against guinea pig CXCR1 and CXCR2 were proved to inhibit the function of these receptors, and were used to elucidate the distinct roles of CXCR1 and CXCR2 on neutrophils. The similarity between human and guinea pig discovered in this work suggests the increased value of the guinea pig, and indicates that the inhibitory antibodies can be used for further clarification of the distinct roles of CXCR1 and CXCR2 by using an in vivo model of a guinea pig, such as a neutrophilic inflammatory disease model.

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Abbreviation

BSA	Bovine serum albumin
E. coli	*Escherichia coli*
ENA-78	Epithelial-derived neutrophil-activating peptide 78
FBS	Fetal bovine serum
GCP-2	Granulocyte chemotactic protein 2
Gp	Guinea pig
GPCR	G-protein coupled receptor
GRO **α**	Growth-related oncogene **α**
IgG	Immunoglobulin G
IL-8	Interleukin-8
NAP-2	neutrophil-activating peptide 2
PBS	Phosphate buffered saline
PEI	Polyethyleneimine
SEM	Standard error of the mean

Chapter 1. Introduction

1.1 CXCR1 and CXCR2, and their ligands

CXCR1 and CXCR2 are seven-transmembrane G protein-coupled receptors (GPCRs) that bind to CXC chemokines as ligands. Both receptors are expressed on various cells, for example, neutrophils, monocytes, CD8+ T cells and NK cells, mast cells, basophils, neurons, keratinocytes, and melanocytes.[1,2] On neutrophils, chemokine receptors, CXCR1 and CXCR2, which are expressed on these cells at similar levels and at higher than on other cell types, mediate chemotaxis, degranulation and the generation of superoxide in respiratory bursts in response to chemokines, and consequently play important roles in preventing the invasion of pathogens (Fig.1-1).[3] CXCR1 and CXCR2 are also involved in chronic inflammation of the lung through continuous neutrophil activation mediated by upregulated chemokines, their common ligand interleukin-8 (IL-8), and CXCR2 -specific ligand growth-related oncogene (GRO)α.[4–7]

Regarding the difference between CXCR1 and CXCR2, these receptors share 76% identity in their amino acid sequences, with the differences being clustered on the N-terminal, fourth transmembrane domain, second extracellular loop, and C-terminal which lead to biological differences such as in internalization and recycling, the associated signaling cascade, and ligand selectivity (Fig.1-2).[1,8,9] In particular, CXCR1

and CXCR2 due to their N-terminals and second extracellular loop, and are thus suggested to play different roles physiologically.[10] In humans, IL-8 (CXCL8) and Granulocyte chemotactic protein 2 (GCP-2, CXCL6) are known to be shared ligands by both CXCR1 and CXCR2,[11,12] while GROα/β/γ (CXCL1/2/3), Epithelial-derived neutrophil-activating peptide-78 (CXCL5), and neutrophil-activating peptide 2 (NAP-2, CXCL7)[13,14] are selective ligands of CXCR2 (Fig.1-3). In inflammation, these chemokines are secreted and upregulated, and the function of CXCR1 and CXCR2 are exerted by the chemokines. This makes it difficult to elucidate their distinct roles under physiological conditions.

1.2 Appropriateness of guinea pig model for CXCR1 and CXCR2 study

To clarify the physiological functions of CXCR1 and CXCR2, it is important to use an animal model in which these receptors and their ligands exhibit homology to those in humans. Mouse, rat, and guinea pig are often utilized as inflammatory animal models,[15,16] but there are interspecies difference among them, especially in CXCR1 and IL-8 (Fig1-4).[17] As for CXCR1, the orthologue of mouse and rat have been controversial for a long time. The rat orthologue of CXCR1 was reported that any ligands did not activate the receptor.[18] The mouse orthologue of CXCR1 was not

functional in the first paper as well as rat,[19] however, Fan et al. and reported subsequently that the receptor transiently expressed on Ba/F3 was functional in response to the ligands,[20] though the expression on the neutrophils has not been demonstrated firmly.[21] By contrast, the guinea pig CXCR1 (gpCXCR1) were identified, and the expression in polymorphonuclear neutrophils were detected. As for IL-8, mice and rats lack an ortholog of IL-8[20,22,23] but, guinea pig IL-8 (gpIL-8) has been identified[24] and was reported to induce the migration of cells expressing either gpCXCR1 or guinea pig CXCR2 (gpCXCR2).[25] Because of the presence of these orthologs and its functional similarity to those in humans, guinea pigs are considered to be an appropriate species to investigate the physiological functions of CXCR1 and CXCR2.

1.3 Inhibitors to CXCR1 and CXCR2

Specific inhibitors against CXCR1 and CXCR2 are indispensable to elucidate the distinct functions of these receptors. As for the small compounds, CXCR2 selective inhibitors and CXCR1 and CXCR2 dual inhibitors have been extensively studied mainly in research on therapies against inflammatory diseases.[26] For example, a comparison between the CXCR2 selective inhibitor SB-656933 and the CXCR1 and

CXCR2 dual inhibitor SCH-527123[27,28] showed that inhibition of both receptors leads to more effective suppression of human neutrophil migration induced by IL-8 or conditioned medium of alveolar macrophage from chronic obstructive pulmonary disease (COPD) patients than CXCR2-specific inhibition *in vitro*.[29] To investigate the specific function of CXCR1 and CXCR2 *in vivo*, these compounds were examined in a guinea pig inflammatory model, but both compound showed cross reactivity to CXCR1 and CXCR2 at similar potency and it was difficult to differentiate their functions.[30]

Inhibitory antibodies against human CXCR1 and CXCR2 have been generated by peptide immunization, which revealed their specific functions *in vitro*. For example, Jones et al. reported that calcium influx of neutrophils induced by IL-8 was mediated through CXCR1 and CXCR2,[31] and Hammond et al. reported that neutrophil migration by GROα was mediated through CXCR2 only, using anti-human CXCR1 and CXCR2 antibodies.[32] These findings are consistent with the ligand selectivity of CXCR1 and CXCR2. In contrast, Hammond et al. also reported that neutrophil migration induced by IL-8 was mediated mainly by CXCR1 and that CXCR2 made only a weak contribution. Antibodies to human CXCR1 and CXCR2 elucidated the distinct function of CXCR1 and CXCR2 *in vitro*, however, the distinct functions of CXCR1 and CXCR2 in inflamed tissue remained unclear because multiple chemokines are involved, and activate and

regulate these receptors synergistically.

1.4 Generation of antibodies against GPCRs

The importance of monoclonal antibodies is increasing rapidly for not only as a tool for specifically inhibiting target molecules but also for therapeutic use. In conventional antibody generation, a purified antigenic protein is used for the immunization of a mouse, however, purification is difficult in the case of multi-transmembrane proteins such as GPCRs or ion channels.[33] To circumvent this problem, peptide immunization of the extracellular domain has been attempted, but there is the risk of discrepancy in peptide conformation compared with the native form, and the efficiency of this approach for obtaining inhibitory antibody is low. Cells expressing the antigen are also utilized, but other antigens on these cells hinder the specific induction of immune response against the antigen.

DNA immunization is a method in which an expression plasmid encoding an antigenic protein is introduced into and expressed on the host cell *in vivo* as an immunogen.[34] This method can overcome the above-mentioned problems because the antigenic protein is expressed with its native conformation and no other protein is not expressed in principle. In DNA immunization, the plasmid can be introduced by various

modified versions of several approaches, for example, intramuscular injection with *in vivo* electroporation,[35,36] intravenous injection using polyethyleneimine (PEI) reagent,[36,37] and intradermal injection with *in vivo* electroporation.[38,39] These DNA immunization methods have been reported to show a higher titer especially against several antigens, but their efficiency levels have yet to be compared.

1.5 Aims and structure of this thesis

This thesis has two main aims. The first is the acquisition of inhibitory antibodies against guinea pig CXCR1 and CXCR2 for the first time. The second is to determine whether these antibodies can be used to elucidate the functional differences between guinea pig CXCR1 and CXCR2.

This thesis consists of four chapters as shown below.

Chapter 1 Introduction

In this chapter, the background of this research is addressed, including CXCR1, CXCR2 and their ligands, interspecies differences in CXCR1 and IL-8, inhibitors of CXCR1 and CXCR2, and antibody acquisition against multi-transmembrane proteins. The aims and structure of this thesis are also introduced.

Chapter 2 Generation of inhibitory antibodies against guinea pig CXCR1 and CXCR2

In this chapter, DNA immunization methods are first characterized, followed by a description of the generation of inhibitory antibodies against guinea pig CXCR1 and CXCR2 are generated by the selected DNA immunization method.

Chapter 3 Elucidation of the distinct roles of guinea pig CXCR1 and CXCR2 in neutrophil migration

This chapter describes elucidation of the distinct roles of CXCR1 and CXCR2 on guinea pig neutrophils using inhibitory antibodies, which were generated as described in chapter 2.

Chapter 4 Conclusions and future work

This chapter provides the conclusions based on the research described in chapters 2 and 3. Future work that could expand this research further is also addressed.

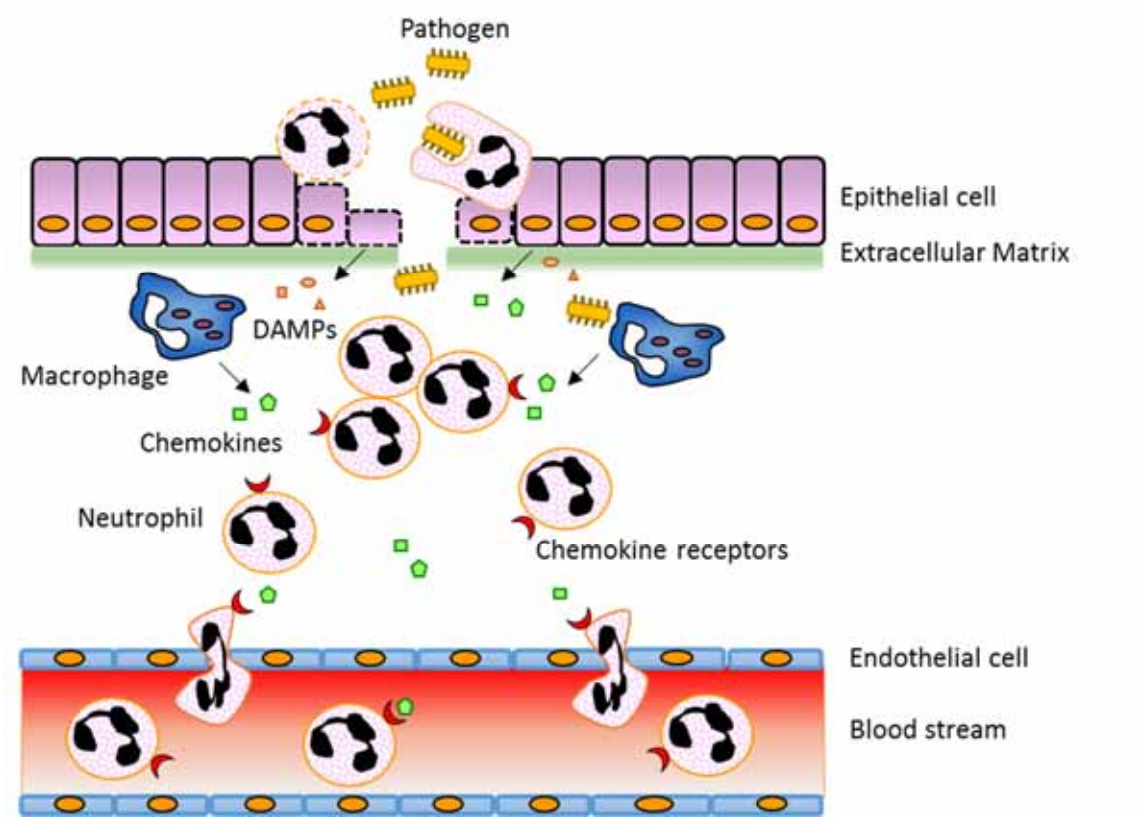

Fig. 1-1 Inflammation and neutrophils in host defense.

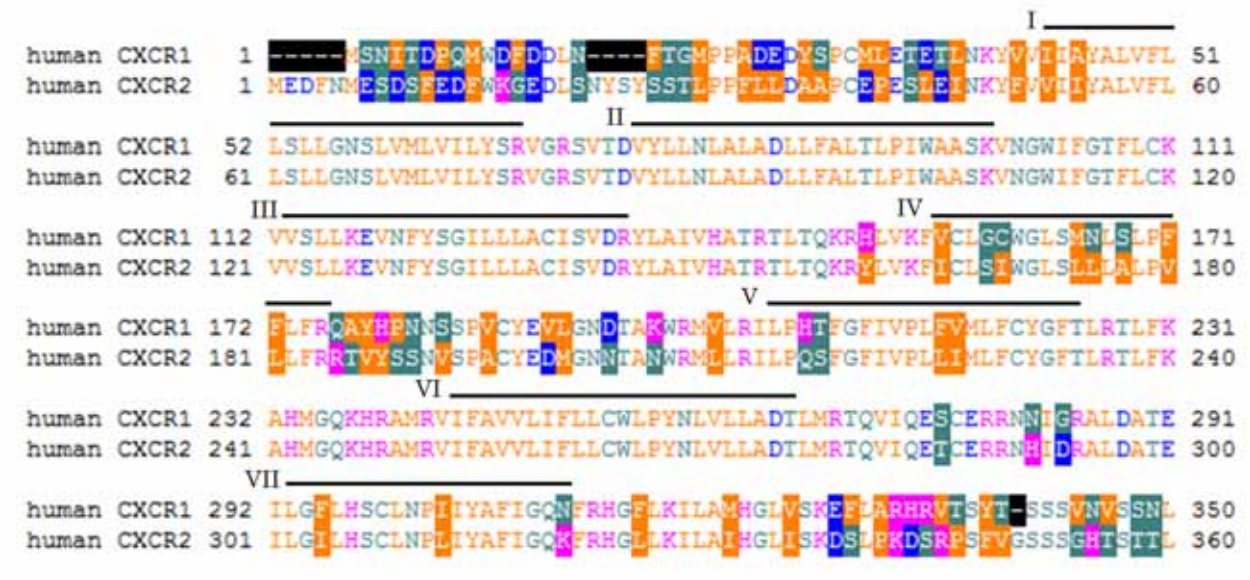

Fig. 1-2 Alignment of human CXCR1 and CXCR2 sequences.

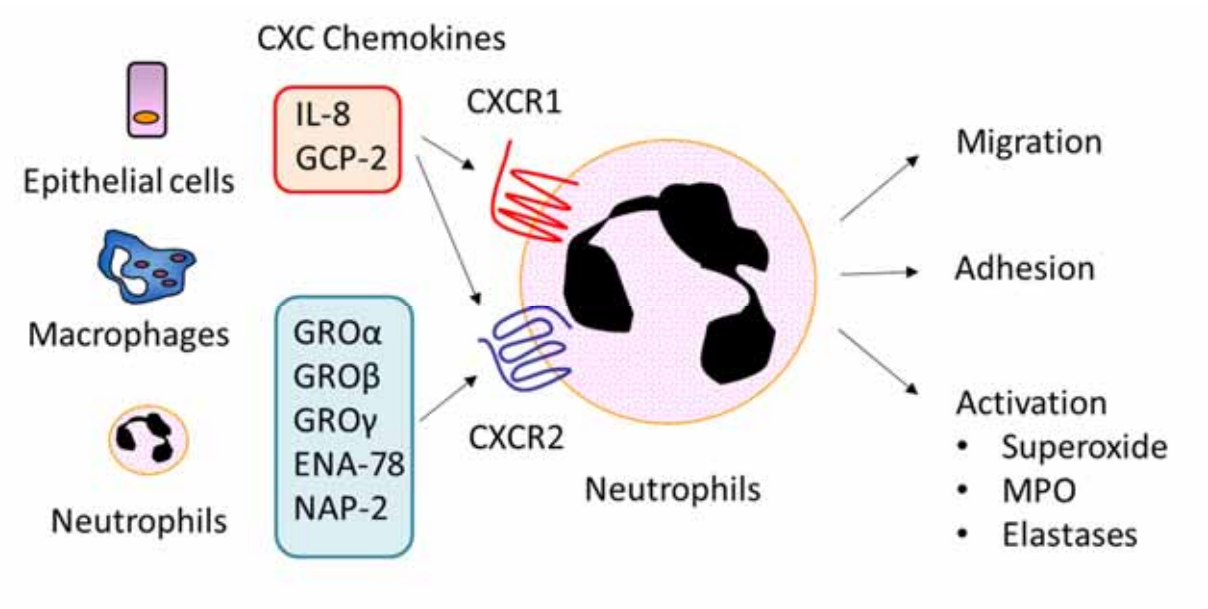

Fig. 1-3 Ligand selectivity of CXCR1 and CXCR2

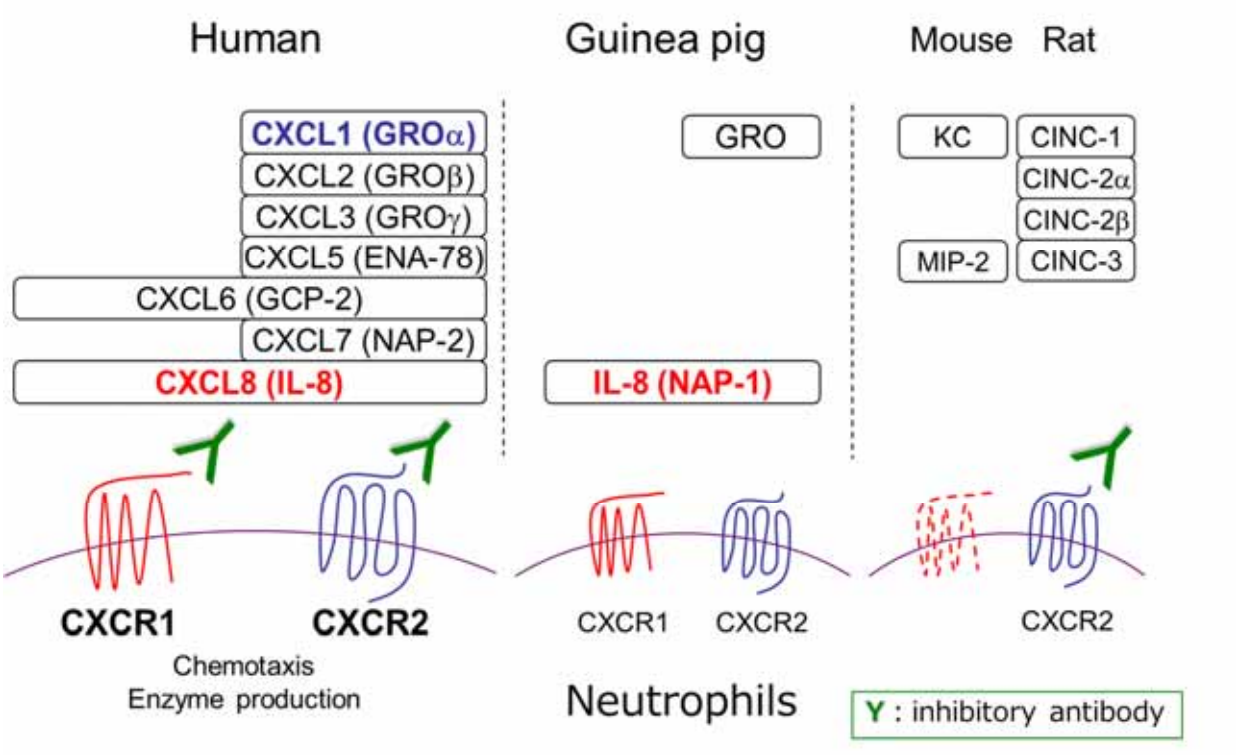

Fig. 1-4 Interspecies difference of CXCR1, CXCR2 and their ligands

Chapter 2. Generation of inhibitory antibodies against guinea pig CXCR1 and CXCR2

2.1 Introduction

CXCR1 and CXCR2 are chemokine receptors that have different selectivity for chemokine ligands, but the distinct roles of each receptor are not fully understood. This is due to the absence of specific inhibitors of them in guinea pigs, which is an appropriate species for investigating CXCR1 and CXCR2 because of their functional similarity to those in humans. In this study, DNA immunization methods were examined because CXCR1 and CXCR2 are multi-transmembrane proteins. Intramuscular injection followed by electroporation was selected as the approach to be used here from the results of a preliminary study comparison the efficacy of different DNA immunization methods, and monoclonal antibodies that specifically bind to guinea pig CXCR1 (gpCXCR1) and guinea pig CXCR2 (gpCXCR2) were generated. To assess the activity of these antibodies, we established CHO-K1 cells stably expressing either gpCXCR1 or gpCXCR2 (CHO/gpCXCR1 or CHO/gpCXCR2). CHO/gpCXCR1 showed migration in response to guinea pig IL-8, and CHO/gpCXCR2 showed migration in response to both guinea pig IL-8 and guinea pig growth-regulated oncogene α. The levels of receptor

selectivity of the chemokines of guinea pigs were the same as those of their human orthologs. The anti-gpCXCR1 and -gpCXCR2 monoclonal antibodies were observed to inhibit cell migration in a concentration-dependent manner. In conclusion, we successfully obtained inhibitory antibodies specific to gpCXCR1 and gpCXCR2. These inhibitory antibodies will be useful to clarify the physiological roles of CXCR1 and CXCR2 in guinea pigs.

2.2 Materials and methods

2.2.1 Expression plasmid construction

Plasmids for the expression of gpCXCR1, gpCXCR2, gpIL-8, and gpGROα were constructed by a standard genetic engineering procedure. In brief, gpCXCR1 (NCBI Refseq: NM_001173416) and gpCXCR2 (NCBI Refseq: NM_001172875) genes were amplified by PCR from a mixture of cDNA from guinea pig lung, skeletal muscle, heart and brain, and inserted into multiple cloning sites of pcDNA3.1(+), resulting in pcDNA3.1/gpCXCR1 and pcDNA3.1/gpCXCR2. For the introduction of a FLAG tag into the N-terminal region of gpCXCR1 and gpCXCR2, both genes were inserted into multiple cloning sites of pFLAG-Myc-CMV-19 (Sigma-Aldrich, St. Louis, MO), resulting in pFLAG-gpCXCR1 and pFLAG-gpCXCR2. GpIL-8 (NCBI Refseq:

NM_001173399) and gpGROα (NCBI Refseq: NM_0011472938) genes were codon-optimized and synthesized in a form linked to a His-tag for protein purification. These genes were inserted into multiple cloning sites of pET11d and pET22b, resulting in pET11d/gpIL-8, pET11d/gpGROα and pET22b/gpGROα.

2.2.2 Mouse immunization and establishment of hybridomas

For DNA immunization, six-week-old female BALB/c mice were used. In order to generate antibodies against native form of the antigens, the expression plasmid without FLAG tag, pcDNA3.1/gpCXCR1 and pcDNA3.1/gpCXCR2 were used. As for intramuscular injection, intramuscular injection of the expression plasmid with hyaluronidase pretreatment followed by *in vivo* electroporation (IM-EP) was examined as reported previously.[35] In brief, after the pretreatment of lower leg muscles with bovine hyaluronidase, 50 μg of expression plasmid was injected into the same site. Two electrode needles were inserted into the same site, and electric pulses (200 V/cm, 50 ms, six times) were delivered with ECM830 (BTX). As for intravenous injection, intravenous injection using in vivo-jetPEI®-Man (Polyplus Transfection, Illkirch, France) (IV-PEI) was used following the manufacturer's instructions. In brief, 50 μg of expression plasmid and the reagent were prepared separately in 5% glucose solution,

and mixed. After incubation for 15 min at room temperature, the complex was injected into tail vein intravenously. As for intradermal injection, intradermal injection of the expression plasmid followed by *in vivo* electroporation (ID-EP) was examined. In brief, after shaving the hair on the back, 50 μg of expression plasmid was injected intradermally. Two electrode needles were then inserted into the same site, and electric pulses (200 V/cm, 50 ms, six times) were delivered with ECM830 (BTX). These DNA immunization protocol were repeated several times every two weeks. Blood samples were collected from the tail vein every two weeks. The antibody response in mouse serum was evaluated by flow cytometry to select a mouse that produced the antibodies against guinea pig CXCR1 and CXCR2 after the DNA immunization.

In order to generate the monoclonal antibody, lymph nodes of the selected mice immunized by IM-EP were harvested, and hybridomas were established by conventional hypoxanthine-aminopterin-thymidine selection and cloning, in accordance with the instructions of ClonaCell HY (Stemcell Technologies, Vancouver, Canada) using SP2 myeloma as a fusion partner. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

2.2.3 Monoclonal antibody production and purification

Culture supernatants of the hybridoma clones were used for flow cytometric screening to pick up clones binding to gpCXCR1 or gpCXCR2. Positive hybridomas were expanded, and the medium was changed to Hybridoma SFM (Thermo Fisher Scientific, Waltham, MA) supplemented with 20% low-immunoglobulin (IgG) fetal bovine serum (FBS). After six days of culturing, the supernatant containing antibodies was collected and filtered. The antibodies were purified using a HiTrap Protein G column (GE Healthcare, Little Chalfont, UK), followed by desalting with a PD-10 column (GE Healthcare). The antibodies were concentrated to approximately 5 mg/ml with Amicon Ultra (Merck Millipore, Darmstadt, Germany). Concentration and purity were determined by High performance liquid chromatography (Agilent Technologies, Santa Clara, CA). Endotoxin level was determined using an Endosafe-PTS (Charles River Laboratories, Wilmington, MA). Isotyping of the antibodies was conducted using the mouse monoclonal antibody isotyping test kit (BIO-RAD, Hercules, CA).

2.2.4 Chemokine production and purification

Plasmids pET11d/gpIL-8, pET11d/gpGROα, and pET22b/gpGROα were transformed into the Origami-B strain or BL21 strain of *Escherichia coli* (*E. coli*). The

expanded transformants were induced to express gpIL-8 and gpGROα using isopropyl β-D-1-thiogalactopyranoside. After overnight culturing at 16ºC or 25ºC, the transformants were collected and lysed. The lysates were purified with HisTrap HP (GE Healthcare). The eluted samples were checked by SDS-PAGE using LReady GELS J Peptide 16.5% (BIO-RAD), followed by desalting with a PD-10 column and objective peak fractionation by reverse-phase chromatography using an ODS-120T column. The samples were lyophilized and dissolved to approximately 50 μM in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin for assaying. The calculated molecular sizes of the recombinant gpIL-8 and gpGROα were 11.1 and 9.7 kDa, respectively. Concentration was determined by the Bradford protein assay, and purity was confirmed by SDS-PAGE. Endotoxin level was determined using Endosafe-PTS.

2.2.5 Cell culture and transfection

HEK293T, The human embryonic kidney cell line HEK293T stably transfected with SV40 large T antigen was purchased from cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ºC and 5% CO_2. The Chinese hamster ovary cell line CHO-K1 was cultured in F-12

supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC and 5% CO_2.

For transient transfection, cells were transfected with an expression plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) following manufacturer's instruction. CHO-K1 cells stably expressing gpCXCR1 or gpCXCR2 were established by the standard method. In brief, these cells were transfected with pcDNA3.1/gpCXCR1 or gpCXCR2 using Lipofectamine 2000. The transfected CHO-K1 cells were then incubated in F-12 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml G418, after which the surviving cells were cloned.

2.2.6 Flow cytometry analysis

The binding activities of mouse serum, hybridoma supernatant, and the anti-gpCXCR1 and gpCXCR2 antibodies were assessed by flow cytometry. Cells expressing gpCXCR1 or gpCXCR2 were detached using TrypLE Express (Thermo Fisher Scientific) and resuspended in staining buffer, PBS supplemented with 5% FBS. The samples were diluted with staining buffer to the indicated concentration. Cells were stained with serum, supernatant, and antibodies for 30 min at 4ºC and washed with staining buffer in each staining step. 1 μg/ml of M2 anti-FLAG tag antibody

(Sigma-Aldrich) was used for the detection of the FLAG tag. Then, 10 μg/ml of FITC-conjugated goat anti-mouse IgG antibody or AlexaFluor488 conjugated goat anti-mouse IgG was used as a secondary antibody. As a control antibody, mouse IgG (Thermo Fisher Scientific) was used. LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (Thermo Fisher Scientific) was used to identify dead cells and exclude them from the analysis. Fluorescence was measured using flow cytometer (FC500: Beckman Coulter, Brea, CA).

2.2.7 Migration assay

CHO-K1, CHO/gpCXCR1 and CHO/gpCXCR2 were detached using TrypLE Express and suspended in assay buffer, DMEM/F-12 without phenol red supplemented with 0.1% bovine serum albumin, at a concentration of 10^5 cells/ml and the indicated concentration of antibodies. GpIL-8 and gpGROα were diluted to the concentrations indicated in the figures with the assay buffer. Prior to the assay, the FluoroBlok™ HTS 24-well multiwell permeable support system with an 8.0-μm high-density polyethylene terephthalate membrane (Corning Inc., Corning, NY) was coated with 10 μg/ml fibronectin. The coated chamber was set onto the 24-well plate, and 250 μl of cell suspension with or without antibody was added to the chamber followed by the addition

of 750 μl of chemoattractant to the lower well of the 24-well plate. The chamber was incubated for 4 h at 37ºC and 5% CO_2. After this incubation, the chamber was stained with 4 μg/ml Calcein AM for 15 min at 37ºC, and the multipoint fluorescence of the underside of the chamber was measured using microplate reader (SpectraMax M3: Molecular Devices LLC, Sunnyvale, CA) at 480 nm excitation and 530 nm emission.

2.3 Results

2.3.1 Comparison of the efficiency of immunization methods

First, the expression of guinea pig CXCR1 and CXCR2 was evaluated to determine whether these GPCRs are expressed transiently on the cell membrane in the DNA immunization. The expression plasmid of pFLAG-gpCXCR1 and pFLAG-gpCXCR2 were transfected transiently into HEK293T (293T/FLAG-gpCXCR1 and 293T/FLAG-gpCXCR2, respectively), and the expression of gpCXCR1 and gpCXCR2 on the transmembrane were detected by binding of anti-FLAG tag antibody using FACS analysis (Fig. 2-1). In respect to the expression efficiency, that of gpCXCR2 was higher than that of gpCXCR1, so gpCXCR2 was selected as a target for comparison among the DNA immunization methods.

The DNA immunization methods examined here were as follows: (i) intramuscular

injection of the expression plasmid with hyaluronidase pretreatment followed by *in vivo* electroporation (IM-EP), (ii) intravenous injection using in vivo-jetPEI®-Man (IV-PEI), (iii) intradermal injection followed by *in vivo* electroporation (ID-EP). In order to evaluate the increase of titer against gpCXCR2, the sera of mice after five times immunization were collected. 1/200 diluted sera of mice immunized by IM-EP, IV-PEI and ID-EP exhibited binding to HEK293T transiently expressing gpCXCR2 (293T/gpCXCR2), and average of mean of fluorescence intensity were 51 (N = 6), 36 (N = 2) and 2 (N = 2), respectively. This result indicated that IM-EP was found to be the most efficient method in three DNA immunization methods (Fig. 2-2), and was thus used for DNA immunization in the subsequent experiments.

2.3.2 Mouse immunization and establishment of hybridoma producing antibodies against guinea pig CXCR1 and CXCR2

To produce anti-gpCXCR1 or anti-gpCXCR2 antibodies, we conducted DNA immunization of BALB/c mice by IM-EP using expression plasmid, pcDNA3.1/gpCXCR1 and pcDNA3.1/gpCXCR2. After this immunization, 1/200 diluted serum of gpCXCR1-immunized and gpCXCR2-immunized mice exhibited binding to HEK293T transiently transfected with pcDNA3.1/gpCXCR1

(293T/gpCXCR1) and pcDNA3.1/gpCXCR2 (293T/gpCXCR2), respectively (Fig. 2-3A, B). Both sera showed specific binding to each antigen. Hybridomas were established using lymph nodes from gpCXCR1-immunized and gpCXCR2-immunized mice, and binding of the supernatants was evaluated against 293T/gpCXCR1 or 293T/gpCXCR2 using flow cytometric analysis. As a result of supernatant screening, hybridoma CR1-002, which produced antibody that bound to 293T/gpCXCR1, and hybridoma CR2-004, which produced antibody that bound to 293T/gpCXCR2, were obtained (Fig. 2-3C, D).

2.3.3 Specificity of binding of antibodies against guinea pig CXCR1 and CXCR2 to cells stably expressing these receptors

The monoclonal antibodies AbCR1 and AbCR2 were purified from supernatants of the hybridoma CR1-002 and CR2-004. The levels of purity of monomeric IgG of AbCR1 and AbCR2 were 97.7 % and 97.8%, respectively. Endotoxin levels were both <0.5 EU/ml, at which no *in vitro* effect of endotoxin could be observed.[40] The isotypes of AbCR1 and AbCR2 were IgG2a and IgG2b, respectively. For the assessment of antibodies, CHO-K1 cells stably expressing either gpCXCR1 (CHO/gpCXCR1) or gpCXCR2 (CHO/gpCXCR2) were established by using pcDNA3.1/gpCXCR1 and

pcDNA3.1/gpCXCR2, respectively. The binding activities of AbCR1 and AbCR2 to CHO-K1, CHO/gpCXCR1 and CHO/gpCXCR2 were evaluated using flow cytometry. Purified AbCR1 and AbCR2 bound only to specific antigens, gpCXCR1 and gpCXCR2, respectively (Fig. 2-4A, B). These findings showed that antibodies that can distinguish gpCXCR1 and gpCXCR2 had been successfully obtained. The histograms representing the AbCR1 and AbCR2 of binding shown in Fig. 2-4C and D suggest that CHO/gpCXCR1 expressed gpCXCR1 heterogeneously and CHO/gpCXCR2 expressed gpCXCR2 homogeneously.

2.3.4 Migration of cells stably expressing guinea pig CXCR1 and CXCR2 in response to guinea pig pIL-8 and growth-related oncogene α

In order to evaluate the activity of the antibodies, we investigated whether CHO/gpCXCR1 and CHO/gpCXCR2 migrate toward gpIL-8 and gpGROα. For this purpose, recombinant gpIL-8 and gpGROα were produced by using Origami-B strain, with the anticipation that disulfide bonds would form efficiently because both chemokine contains two disulfide bonds. First, pET11d vectors were used for chemokine expression. Here, gpIL-8 production was observed upon overnight culture at 25ºC after the induction of expression, however, gpGROα production was not (Fig.

2-5A). This low productivity of gpGROα was consistent to the previous report. To improve the productivity, the gpGROα gene was subcloned to pET22b, including its sequence that acts as a signal for its localization to the periplasm with the aim of achieving the efficient formation of disulfide bonds in the neutrophilic conditions that prevail in the of periplasm.[41] As a result of the transfection of pET22b into Origami-B followed by overnight culture at 25ºC after the induction of expression, gpGROα expression was successfully observed (Fig. 2-5B). The molecular sizes of gpIL-8 and gpGROα were estimated to be 11 and 10 kDa, respectively, in SDS-PAGE under reducing conditions, which matched the calculated molecular weight (data not shown). No band except the objective protein was observed in the lanes loaded with the IL-8 and GROα samples. Endotoxin levels were both <0.5 EU/ml. In the migration system featuring a Boyden chamber coated with fibronectin, gpIL-8 induced the migration of CHO/gpCXCR1 in a concentration-dependent manner from a concentration of 4 nM, but gpGROα did not (Fig. 2-6A, B) even at 100 nM. In contrast, both gpIL-8 and gpGROα induced the migration of CHO/gpCXCR2 in a concentration-dependent manner from a concentration of 4 nM (Fig. 2-6C, D). The receptor selectivity of gpGROα was found to be consistent with the human GROα. In contrast, parental CHO-K1 cells did not migrate in response to gpIL-8 or gpGROα (data not shown).

These results indicate that the inhibitory activity of anti-gpCXCR1 or -gpCXCR2 antibodies can be evaluated using a system involving migration in response to gpIL-8, which induced the migration of both CHO/gpCXCR1 and CHO/gpCXCR2, or a system of involving migration in response to gpGROα, which induced migration of only CHO/gpCXCR2, due to the homology of these receptors and their ligands with those in humans.

2.3.5 Inhibitory activity of antibodies against guinea pig CXCR1 and CXCR2 in migration assay

The inhibitory activities of AbCR1 and AbCR2 were evaluated in migration assays using CHO/gpCXCR1 and CHO/gpCXCR2. Chemotactic responses of the cells were induced by gpIL-8 or gpGROα at a concentration at which we observed migration at a sufficient level to evaluate the inhibitory activity of the antibodies. AbCR1 inhibited CHO/gpCXCR1 migration induced by 10 nM gpIL-8 in a concentration-dependent manner (Fig. 2-7A). AbCR2 inhibited CHO/gpCXCR2 migration induced by 10 nM gpIL-8 and also inhibited the cell migration induced by 30 nM gpGROα in a concentration-dependent manner (Fig. 2-7B, C). The control mouse IgG did not show inhibitory effects on the migration of CHO/gpCXCR1 or CHO/gpCXCR2. These results

indicate that AbCR1 and AbCR2 possess inhibitory activity for each specific antigen, gpCXCR1 and gpCXCR2, respectively.

2.4 Summary and discussion

This study had the aim of acquiring inhibitory monoclonal antibodies specific to gpCXCR1 and gpCXCR2. First, intramuscular injection followed by electroporation was selected as DNA immunization method as a result of comparison of three DNA immunization methods. By this method, DNA immunization of mice was conducted, and AbCR1 and AbCR2, monoclonal antibodies specific to gpCXCR1 and gpCXCR2 were selected. The inhibitory activity of AbCR1 and AbCR2 was further demonstrated by establishing cell migration assays in which gpIL-8 induced chemotactic responses of CHO/gpCXCR1 and CHO/gpCXCR2 or gpGROα induced chemotactic responses of CHO/gpCXCR2. As far as I know, this is the first report to present anti-gpCXCR1 and gpCXCR2 monoclonal antibodies with inhibitory activity.

Although no reports have described the acquisition of anti-gpCXCR1 and gpCXCR2 antibodies, regarding their human equivalents, anti-hCXCR1 or hCXCR2 antibodies, some reports have presented their inhibitory effect on hCXCR1 or hCXCR2.[31,32,42,43)] To investigate whether anti-hCXCR1 or -hCXCR2 antibodies would

bind to gpCXCR1 or gpCXCR2, four anti-hCXCR1 antibodies and five anti-hCXCR2 antibodies available from commercial sources were collected and their binding to CHO/gpCXCR1 or gpCXCR2 were assessed, but none of the antibodies exhibited binding. Moreover, these anti-gpCXCR1 and gpCXCR2 antibodies did not show binding to hCXCR1 or hCXCR2. The levels of sequence identity of the extracellular domain of CXCR1 and CXCR2 between humans and guinea pigs are 53% and 53%, respectively (Fig. 2-8), which probably explain to discrepancy in binding between humans and guinea pigs.

The study of cell migration assays using recombinant gpIL-8 and gpGROα revealed consistency between humans and guinea pigs in terms of the chemokine ligand specificity of CXCR1 and CXCR2. In this study, gpIL-8 induced the migration of both CHO/gpCXCR1 and CHO/gpCXCR2. In contrast, gpGROα induced the migration of only CHO/gpCXCR2. These results are consistent with the findings in studies of human CXCR1 and CXCR2. Takahashi et al. previously reported that gpIL-8 induced the migration of HEK293 cells stably expressing gpCXCR1 and also HEK293 cells expressing gpCXCR2, but they could not show migration concerning gpGROα because of its unavailability.[25)] Thus this results are the first to demonstrate the functional validity of gpCXCR2 in the migratory response toward gpGROα. These results indicate

that AbCR1 and AbCR2 are authentic inhibitory antibodies against gpCXCR1 and gpCXCR2.

The inhibitory effect of AbCR1 on chemotactic responses of CHO/gpCXCR1 toward gpIL-8 and that of AbCR2 on chemotactic responses of CHO/gpCXCR2 toward gpIL-8 were both partial. One possible reason for this is that the antibody concentration was not sufficient to inhibit the migration completely. However, the concentration of the antibodies could not be increased due to the limited of concentration of stock antibody. Alternatively, another plausible reason for this partial inhibitory effect is a difference in the epitopes between antibodies and ligands. Modified gpCXCR1 and gpCXCR2 whose extracellular loops are substituted with the corresponding extracellular loops of human ones, should be useful for epitope identification, as indicated by a previous report that attempted to define the epitope of a chemokine to its receptor.[44] This examination of epitopes remains a subject for further study on anti-gpCXCR1 and -gpCXCR2 antibodies.

Specific antibodies that can distinguish gpCXCR1 and gpCXCR2 are helpful to elucidate the functions of CXCR1 and CXCR2, especially in neutrophilic respiratory diseases because guinea pigs are often utilized as respiratory disease models.[15] The expression patterns of CXCR1 and CXCR2 can be determined using these antibodies.

The physiological roles of CXCR1 and CXCR2 can also be elucidated. To date, a variety of small compounds that inhibit CXCR1/CXCR2 have been studied, but no group has clarified the contribution of CXCR1 to neutrophilic disease because of the absence of IL-8 in typical animal models based on mice and rats. However, recently, Planagumà et al. evaluated the inhibitory activity of these selective antagonists in guinea pigs to elucidate the function of CXCR1. They found that these compounds suppressed the functions of both gpCXCR1 and gpCXCR2 at similar levels due to the interspecies difference, but they failed to elucidate the contribution of gpCXCR1.[30] The anti-gpCXCR1 and -gpCXCR2 antibodies should overcome this hurdle because they show clear specificity to each receptor.

In summary, specific antibodies against gpCXCR1 and gpCXCR2 with exhibiting the ability to inhibit the activities of these receptors were successfully obtained. These antibodies will be valuable for elucidating the physiological roles of CXCR1 and CXCR2 in guinea pigs.

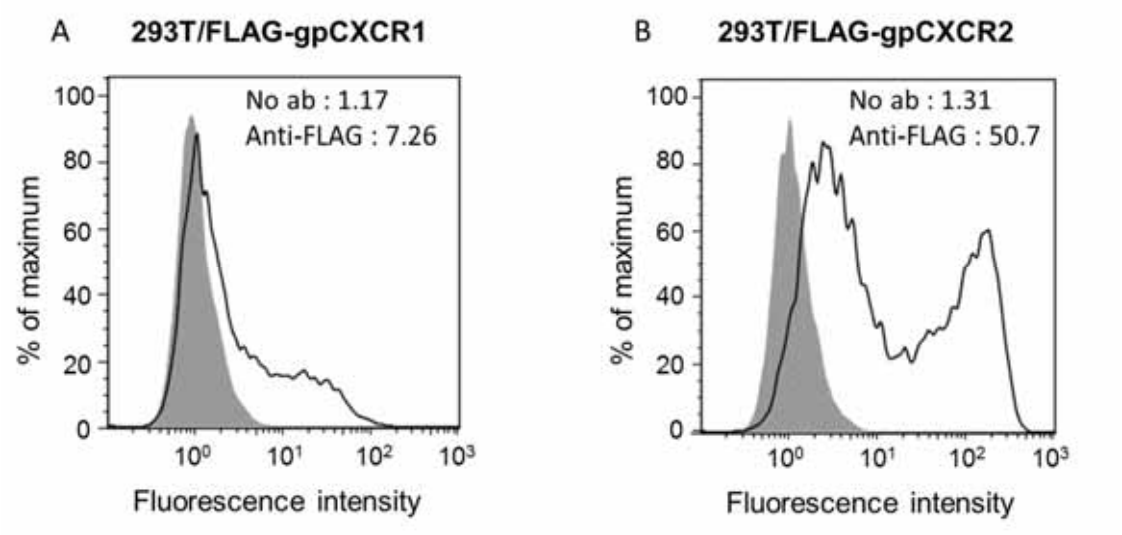

Fig. 2-1 Transient expression of guinea pig CXCR1 and CXCR2.

Binding of anti-FLAG tag antibody (solid line) or no antibody (grey filled) to **A)** 293T/FLAG-gpCXCR1 and **B)** 293T/FLAG-gpCXCR2. Mean of fluorescence intensity was written in the figure.

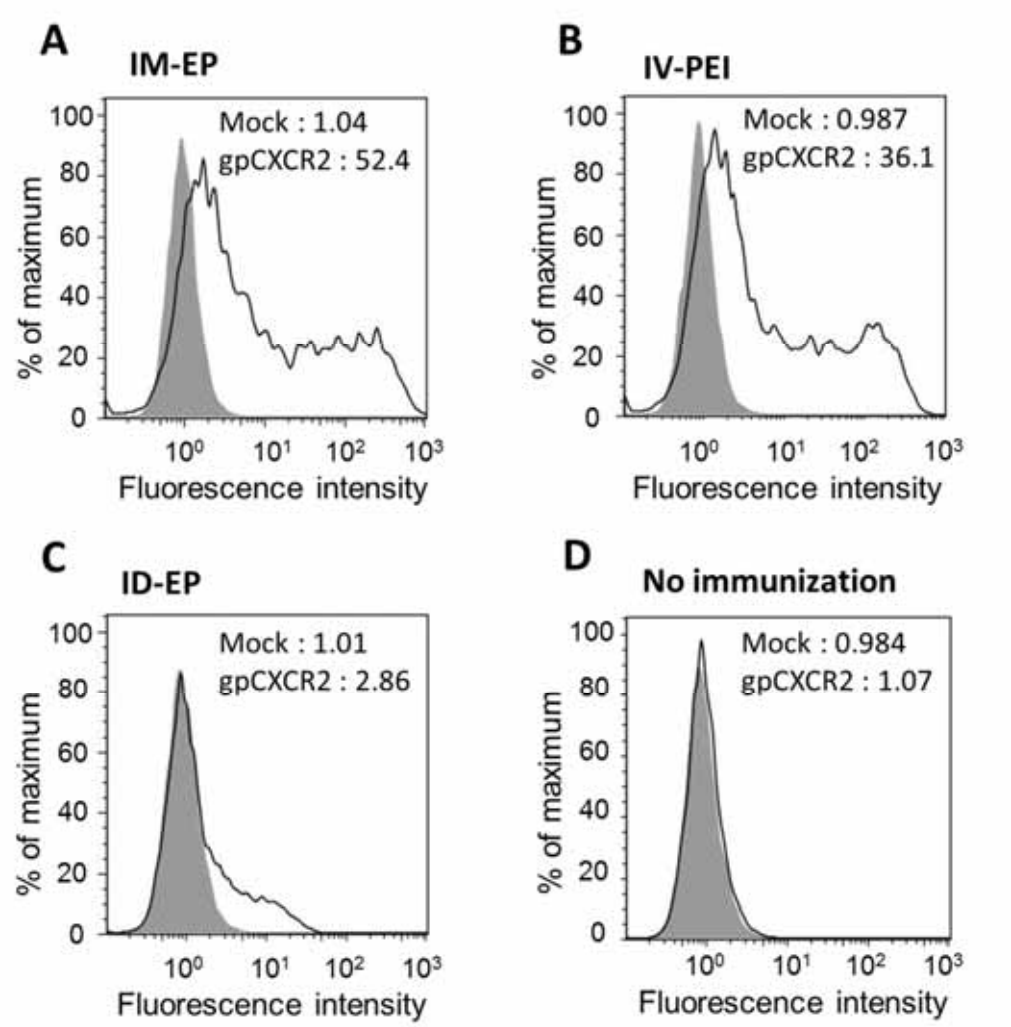

Fig. 2-2 Titer comparison of sera from gpCXCR2-immunized mice by DNA immunization methods.

Binding of 1/200 diluted serum from gpCXCR2-immunized mouse by **A)** intramuscular injection followed by electroporation, **B)** intravenous injection using in vivo jetPEI-Man, **C)** intradermal injection followed by electroporation, and **D)** negative control to 293T/gpCXCR2 (solid line) and 293T/mock (gray filled). Mean of fluorescence intensity was written in the figure.

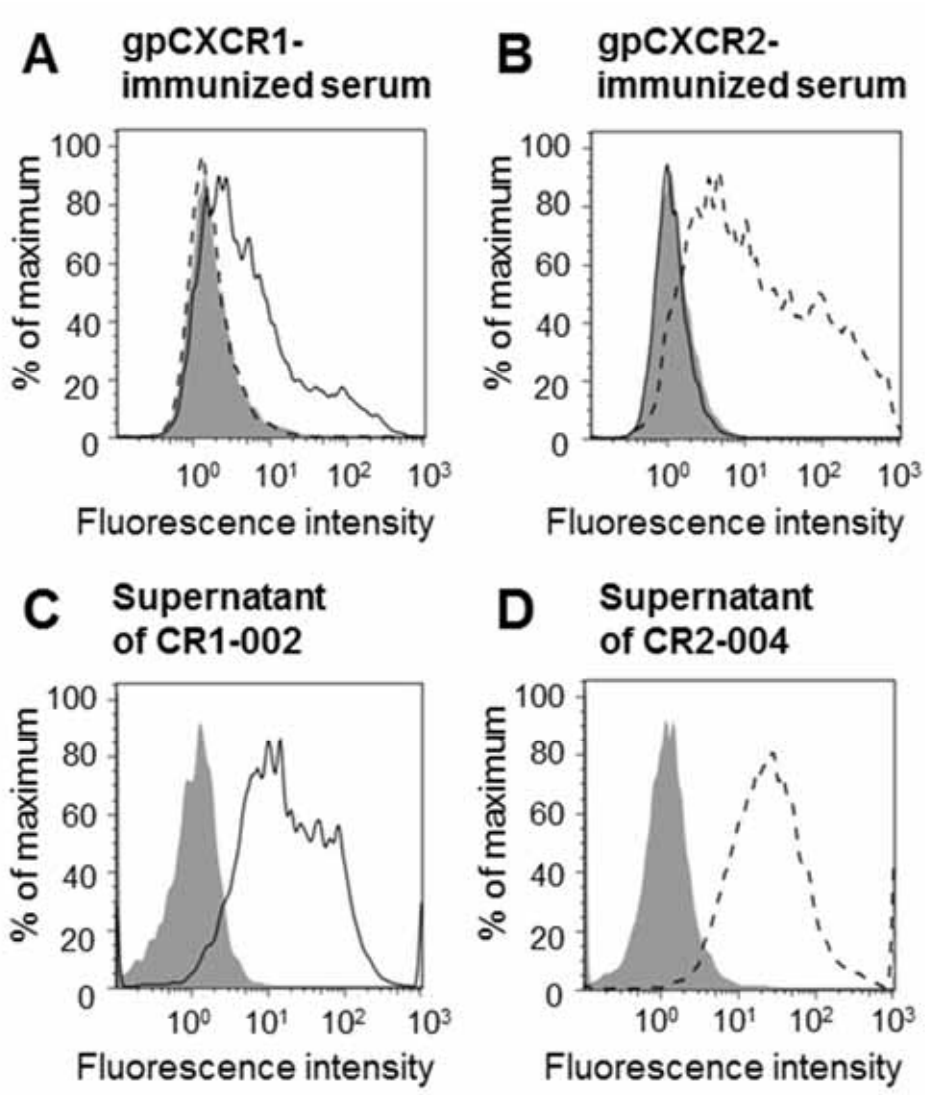

Fig. 2-3 Binding of sera from immunized mice and hybridoma supernatants to cells transiently expressing gpCXCR1 or gpCXCR2.

Binding of 1/200 diluted serum from **A)** gpCXCR1-immunized mouse and **B)** gpCXCR2-immunized mouse to 293T/mock (gray filled), 293T/gpCXCR1 (solid line), and 293T/gpCXCR2 (dashed line). Binding of supernatant of **C)** CR1-002 and **D)** CR2-004 to 293T/mock (gray filled), 293T/gpCXCR1 (solid line), and 293T/gpCXCR2 (dashed line).

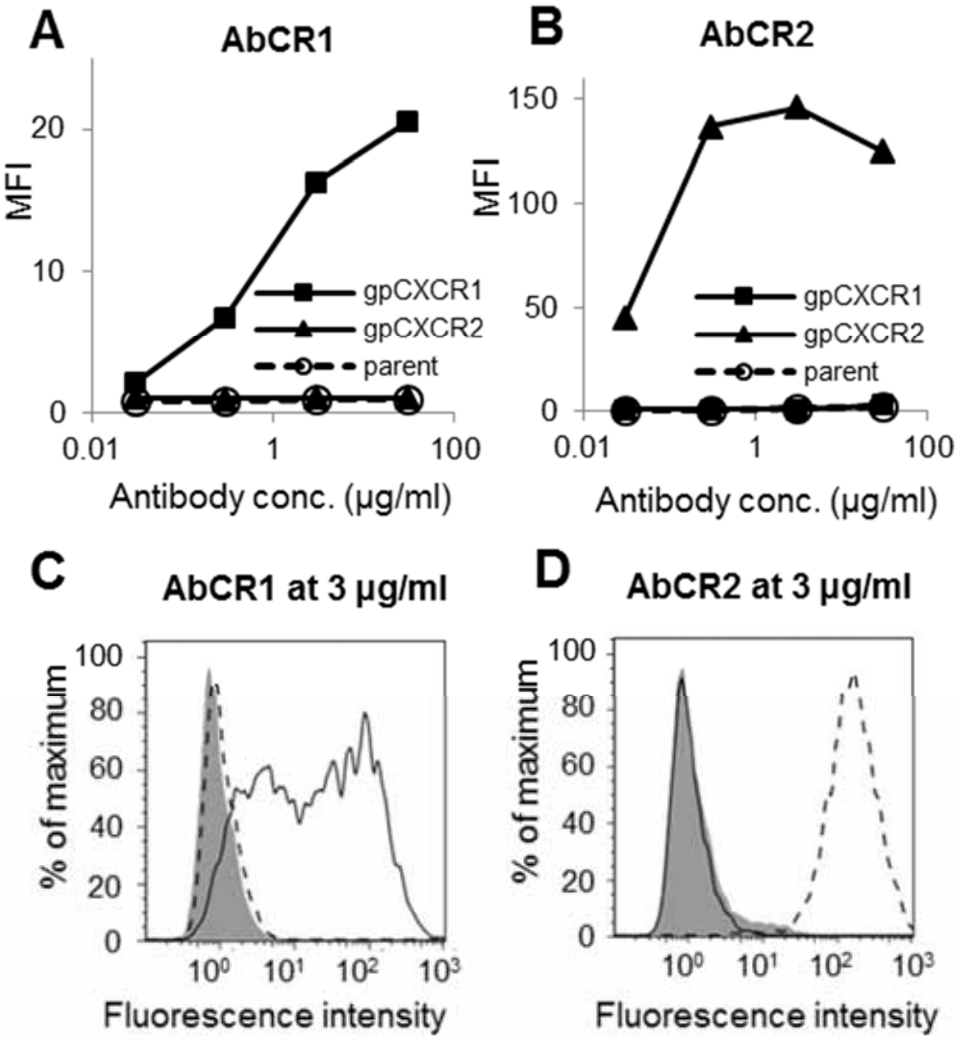

Fig. 2-4 Binding of anti-gpCXCR1 and -gpCXCR2 antibodies to cells stably expressing gpCXCR1 or gpCXCR2.

Binding of **A)** anti-gpCXCR1 antibody AbCR1 and **B)** anti-gpCXCR2 antibody AbCR2 at the indicated concentrations to CHO-K1 (open circle with dashed line, indicated as parent), CHO/gpCXCR1 (filled square with solid line, indicated as gpCXCR1), and CHO/gpCXCR2 (filled triangle with solid line, indicated as gpCXCR2) are presented. Binding of **C)** AbCR1 and **D)** AbCR2 at 3 μg/ml to CHO-K1 (gray filled), CHO/gpCXCR1 (solid line) or CHO/gpCXCR2 (dashed line).

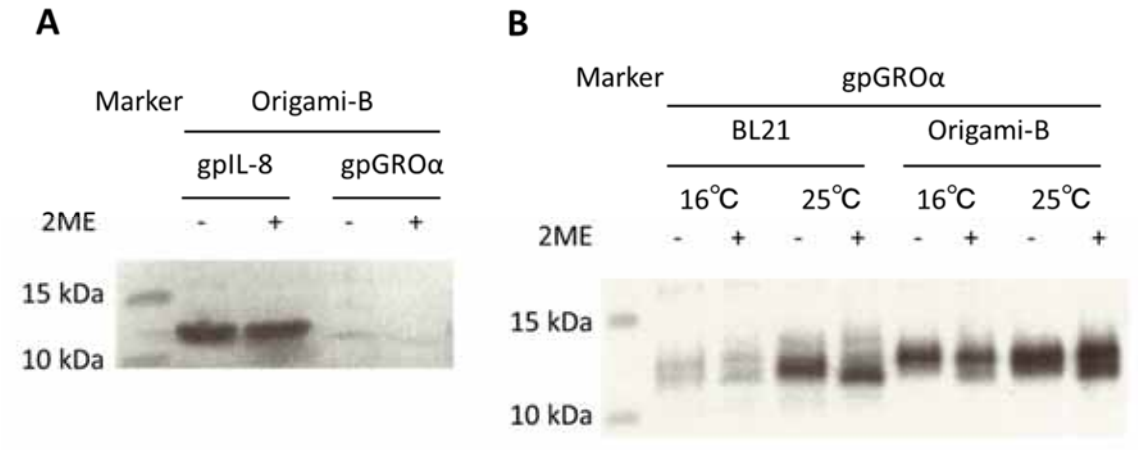

Fig. 2-5 Evaluation of chemokine production by SDS-PAGE.

A) A total of 10 μl of His-tag purified sample derived from pET11d/gpIL-8- or pET11d/gpGROα-transfected Origami-B cultured overnight at 25ºC was loaded and stained by Coomassie dye, **B)** A total of 10 μl of His-tag purified sample derived from pET22b/gpGRO α -transfected BL21 or Origami-B cultured overnight at 16ºC or 25ºC were loaded and stained by Coomassie dye.

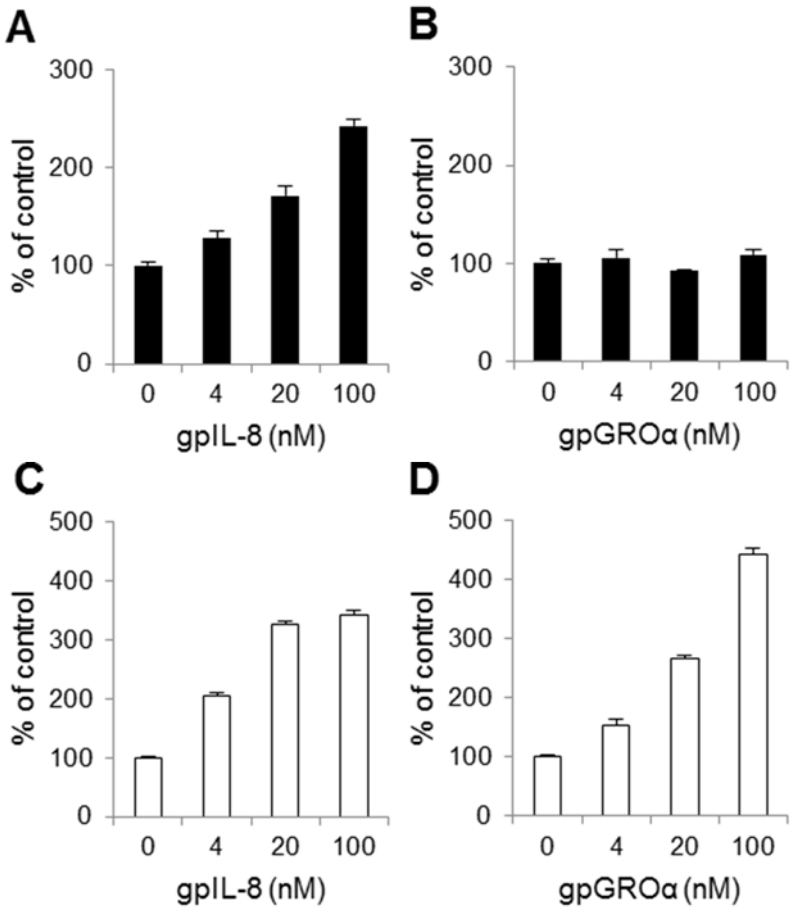

Fig. 2-6 Migration of cells stably expressing either gpCXCR1 or gpCXCR2 induced by chemokine ligand.

Migration of CHO/gpCXCR1 (closed bar) induced by **A)** gpIL-8 and **B)** gpGROα. Migration of CHO/gpCXCR2 (open bar) induced by **C)** gpIL-8 and **D)** gpGROα. The cells were cultured for 4 h in a Boyden chamber. The migrated cells in the underside of the chamber were measured. Migrated cells without chemokine induction were set to 100% as a control. The data are presented as the percent of migration relative to the control with standard error of the mean (SEM) (n=3).

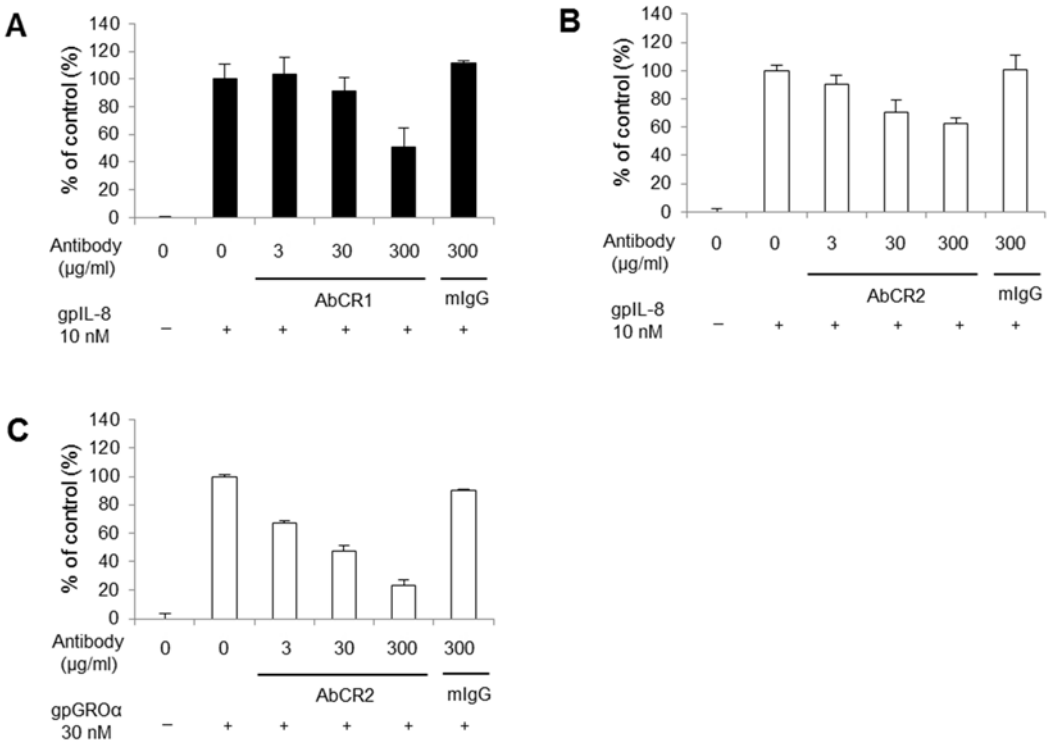

Fig. 2-7 Inhibitory activity of anti-gpCXCR1 and -gpCXCR2 antibodies against migration of cells expressing either gpCXCR1 or gpCXCR2.

A) Migration of CHO/gpCXCR1 (closed bar) was induced by 10 nM IL-8 in the presence of AbCR1 at indicated concentrations. Migration of CHO/gpCXCR2 (open bar) was induced by **B)** 10 nM IL-8 or **C)** 30 nM GROα in the presence of AbCR2 at the indicated concentrations. The migration of cells induced by chemokine in the absence of antibody was set to 100% as control. The data are presented as the percent of migration relative to the control with SEM (n=3).

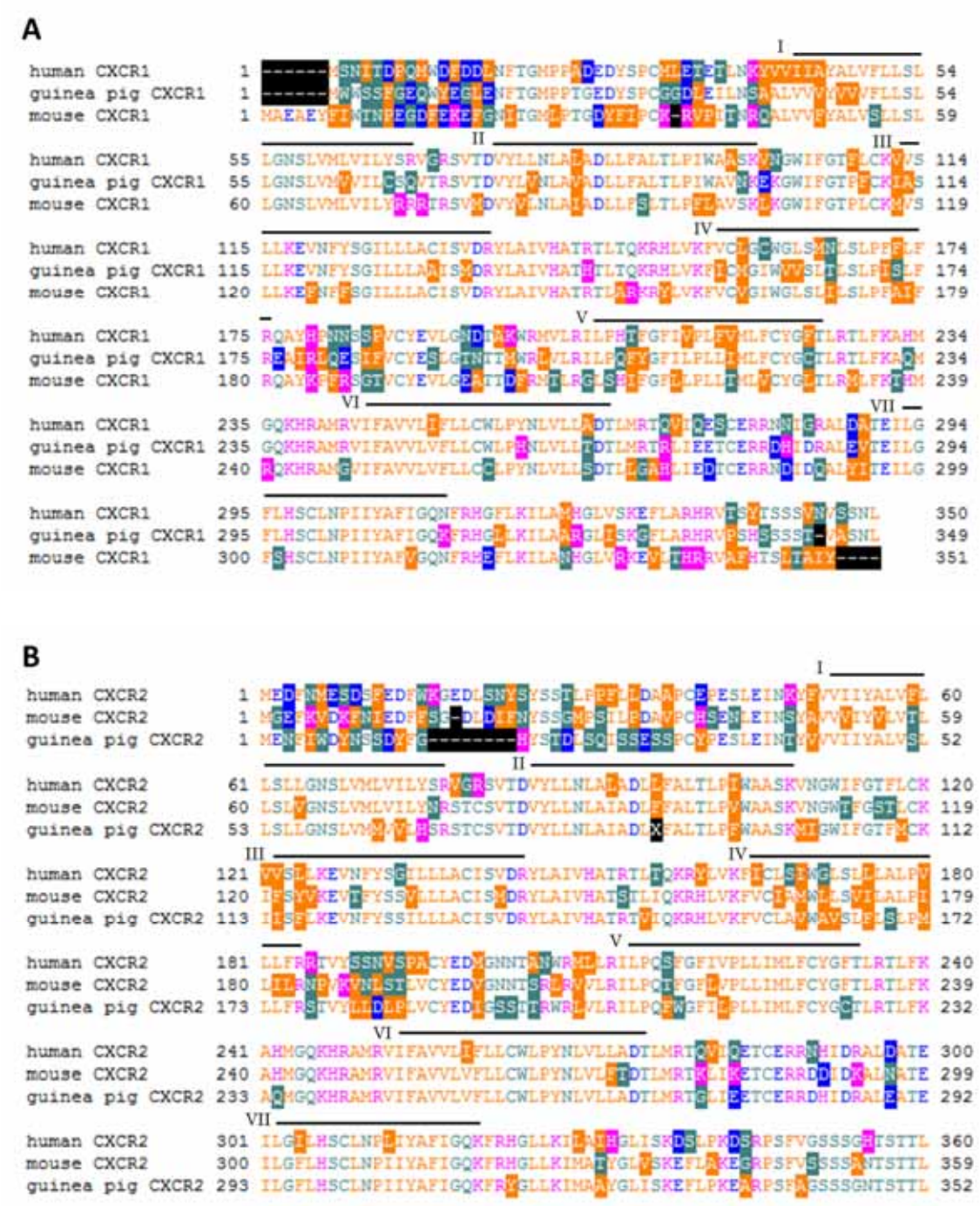

Fig. 2-8 Alignment CXCR1 and CXCR2 of human, guinea pig and mouse sequences.

A) CXCR1 and **B)** CXCR2 of human, guinea pig and mouse sequences are depicted. The position of the seven transmembrane domains are overlined. Mismatched amino acids were reversed.

Chapter 3. Elucidation of the distinct roles of guinea pig CXCR1 and CXCR2 in the neutrophil migration

3.1 Introduction

The chemokine receptors CXCR1 and CXCR2 are conserved between guinea pigs and humans, but the distinct role of each receptor in the chemotactic responses of neutrophils against chemokine ligands have remained unclear, due in part to the lack of specific inhibitors against these receptors in guinea pigs. To bridge this research gap, in this study, the roles of guinea pig CXCR1 and CXCR2 on neutrophils in chemotactic responses to guinea pig IL-8 and GROα were investigated by using specific inhibitory antibodies against these receptors. Neutrophil migration induced by IL-8 was partially inhibited by either anti-CXCR1 antibody or anti-CXCR2 antibody. The migration was inhibited completely when both anti-CXCR1 and anti-CXCR2 antibodies were combined. In contrast, neutrophil migration induced by GROα was not inhibited by anti-CXCR1 antibody, while it was inhibited profoundly by anti-CXCR2 antibody. These results indicate that CXCR1 and CXCR2 mediated migration induced by IL-8 synergistically and only CXCR2 mediates migration induced by GROα in guinea pig neutrophils. The findings on the ligand selectivity of CXCR1 and CXCR2 in guinea pigs are thus consistent with those in humans.

3.2 Materials and methods

3.2.1 Production of antibodies and chemokines

Anti-gpCXCR1 and gpCXCR2 monoclonal antibodies, AbCR1 (mouse IgG2a antibody) and AbCR2 (mouse IgG2b antibody) were generated by the DNA immunization of mice as described in chapter 2. Mouse IgG2a isotype control antibody and mouse IgG2b isotype control antibody were purchased from Thermo Fisher Scientific.

Guinea pig IL-8 and GROα were produced as described in chapter 2.

3.2.2 Preparation of guinea pig neutrophils

Five- to seven-weeks-old female Hartley guinea pigs were used to obtain neutrophils. In brief, the guinea pigs were euthanized using CO_2 gas and their lower limbs were dislocated. Skin and muscle were removed from femurs and tibias, and they were separated from the lower limbs. After rinsing with RPMI1640 supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin, the ends of the bones were cut and bone marrow cells were recovered by flushing both ends of the bone shafts with 10 ml of RPMI1640 supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin using a 25-gauge needle and a 5-ml syringe.

Neutrophils were separated from bone marrow cells by density gradient centrifugation. In brief, 10 ml of Histopaque 1119 (Sigma-Aldrich) was added to a 50-ml conical tube, and 10 ml of Histopaque 1077 (Sigma-Aldrich) and 25 ml of the recovered bone marrow cells were overlaid sequentially without disturbing the interfaces between each layer. The tube was centrifuged for 30 min at 2200 rpm and 25ºC without brake. After this centrifugation, neutrophils were collected from the interface between Histopaque 1119 and Histopaque 1077 layers, and washed and resuspended with RPMI1640 supplemented with 10% FBS and 100 U/ml penicillin, and 100 μg/ml streptomycin. In the separated cells, the population of neutrophils was determined by the flow cytometry analysis to be 40% to 50%.

All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

3.2.3 Flow cytometry analysis

The expression of gpCXCR1 and gpCXCR2 on the neutrophils was assessed by flow cytometry. Separated guinea pig neutrophils were resuspended in staining buffer, PBS supplemented with 5% FBS. The antibodies were diluted with the staining buffer

to the indicated concentration. The cells were incubated with antibody solutions for 30 min at 4ºC, followed by washing with staining buffer. They were then stained with 10 μg/ml Alexa488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific) for 30 min at 4ºC. For the exclusion of dead cells, a LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (Thermo Fisher Scientific) was used. Fluorescence of the neutrophils was measured using a flow cytometer (FC500; Beckman Coulter) and analyzed by analysis software (FlowJo; FlowJo LLC).

3.2.4 Migration assay

Separated guinea pig neutrophils were suspended in assay buffer, RPMI supplemented with 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at a concentration of 2 x 10^7 cells/ml and the indicated concentration of antibodies. GpIL-8 and gpGROα were diluted to the indicated concentrations with the assay buffer as a chemoattractant. A Transwell® with 5.0 μm Pore Polycarbonate Membrane Insert was set onto a 24-well plate, and 100 μl of cell suspension was added to the insert followed by the addition of 600 μl of chemoattractant to the lower well. The plate was incubated for 90 min at 37ºC and 5% CO_2. After the incubation, the inserts were removed and 0.1% glutaraldehyde was added to the lower well for immobilization. Flow Count

Fluorospheres (Beckman Coulter) were added to the lower well and the number of migrated neutrophils was counted using an FC500 flow cytometer.

3.3 Results

3.3.1 GpCXCR1 and gpCXCR2 expression on guinea pig neutrophils

To analyze the expression profile of gpCXCR1 and gpCXCR2 on the guinea pig neutrophils, the binding of anti-gpCXCR1 antibody and anti-gpCXCR2 antibody, AbCR1 and AbCR2, was evaluated. Guinea pig neutrophils were incubated with each antibody and the bound antibodies were detected by flow cytometry. Both AbCR1 and AbCR2 bound to the neutrophils in a concentration-dependent manner, and the bindings of the antibodies saturated at 300 μg/ml (Fig. 3-1A). In addition, the histograms showed shifts in a single peak by AbCR1 (Fig. 3-1B) and AbCR2 (Fig. 3-1C) against neutrophils. These results indicated that the guinea pig neutrophils express both gpCXCR1 and gpCXCR2 homogeneously at the protein level.

3.3.2 Chemotactic activity of gpIL-8 and gpGROα against guinea pig neutrophils

The migration of the neutrophils toward gpIL-8 and gpGROα was evaluated in order to determine the chemotactic activity of these chemokines against the guinea pig

neutrophils. For migration assays of the guinea pig neutrophils, a Transwell® system was used in which the neutrophils migrate toward lower wells filled with chemoattractant across a porous membrane insert. The number of migrated neutrophils in the lower well was counted by flow cytometry. The results showed that both gpIL-8 and gpGROα induced the migration of guinea pig neutrophils in a concentration-dependent manner, and both migrations peaked at 10 nM gpIL-8 and 10 nM gpGROα (Fig. 3-2A, B).

3.3.3 Contribution of gpCXCR1 and gpCXCR2 to the migration of guinea pig neutrophils

The activity of AbCR1 and AbCR2 regarding inhibiting the migration of guinea pig neutrophils toward gpIL-8 and gpGROα in migration assays was evaluated by using a Transwell® to elucidate the contributions of the gpCXCR1 and gpCXCR2 to this. In the following assays, chemotactic responses of the neutrophils were induced by 5 nM gpIL-8 or 10 nM gpGROα, which were concentrations that were applied in order to ensure sufficient migration to evaluate the inhibitory activity of the antibodies. Regarding the migration induced by gpIL-8, both AbCR1 and AbCR2 showed partial inhibition of neutrophil migration (Fig. 3-3A, B). Additionally, the combination of

AbCR1 and AbCR2, both at 100 μg/ml, showed synergistic inhibition of the migration induced by gpIL-8 (Fig. 3-3C), which is a sufficient concentration for the occupation of CXCR1 and CXCR2 on the neutrophils according to Fig. 1. On the other hand, the migration induced by 10 nM gpGROα was not affected at all, even at a concentration of AbCR1 of 100 μg/ml, but was significantly inhibited at 100 μg/ml AbCR2 (Fig. 3-3D, E). These results indicate that both gpCXCR1 and gpCXCR2 synergistically mediate the migration induced by gpIL-8, but only gpCXCR2 mediates the migration induced by gpGROα.

3.4 Summary and discussion

In this study, specific antibodies were used to clarify the roles of CXCR1 and CXCR2 in the migration of guinea pig neutrophils. First, it was demonstrated that the neutrophils expressed both CXCR1 and CXCR2 homogeneously, and IL-8 and GROα induced migration of the neutrophils. The neutrophil migration induced by IL-8 was inhibited partially by anti-CXCR1 or -CXCR2 antibodies, and completely by the combination of these antibodies. This indicates that both receptors are involved in the chemotactic responses of neutrophils towards IL-8 and that IL-8 signals are mediated through only these two receptors, while the neutrophil migration induced by GROα was

inhibited by only the anti-CXCR2 antibody (Fig. 3-4).

This is the first demonstration that the distinct roles of CXCR1 and CXCR2 on the guinea pig neutrophils were by using inhibitory antibodies specific for these receptors. As for small-molecule inhibitors, Planagumà et al. reported that four selective inhibitors of human CXCR2 were found to inhibit gpCXCR1 and gpCXCR2 with similar potency due to the interspecies difference.[30] Anti-human CXCR1 and CXCR2 antibodies have been reported, but, no antibodies were found to cross-react with either guinea pig CXCR1 or CXCR2 due to the low homology between humans and guinea pigs. Therefore, there were no specific inhibitors against gpCXCR1 and gpCXCR2 other than AbCR1 and AbCR2, and the use of these antibodies distinctly showed for the first time the chemotactic functions of CXCR1 and CXCR2.

This finding that guinea pig neutrophils express CXCR1 and CXCR2 and respond to IL-8 and GROα reinforces the usefulness of guinea pigs as animal models for studying neutrophilic inflammatory diseases. In human neutrophil, the functions of CXCR1 and CXCR2 have been evaluated by using anti-human CXCR1 and CXCR2 antibodies. Similar to these results, Jones et al. demonstrated that the calcium influx of neutrophils induced by IL-8 was mediated through CXCR1 and CXCR2,[31] and Hammond et al. demonstrated that the migration of neutrophils induced by GROα was mediated through

CXCR2 only.[32] In contrast, the group also reported that the migration of neutrophil induced by IL-8 was mediated mainly by CXCR1 and that CXCR2 made only a small contribution. These results might be dependent on the concentration used in the IL-8 assay and the affinity of the antibodies. Further study is needed to determine which receptor works preferentially against IL-8 in guinea pigs and humans.

In summary, these results indicate that both CXCR1 and CXCR2 function on the neutrophils of guinea pigs in response to their ligands, similarly to how they do in humans. This further suggested the value of the guinea pig as a model of neutrophilic inflammatory disease.

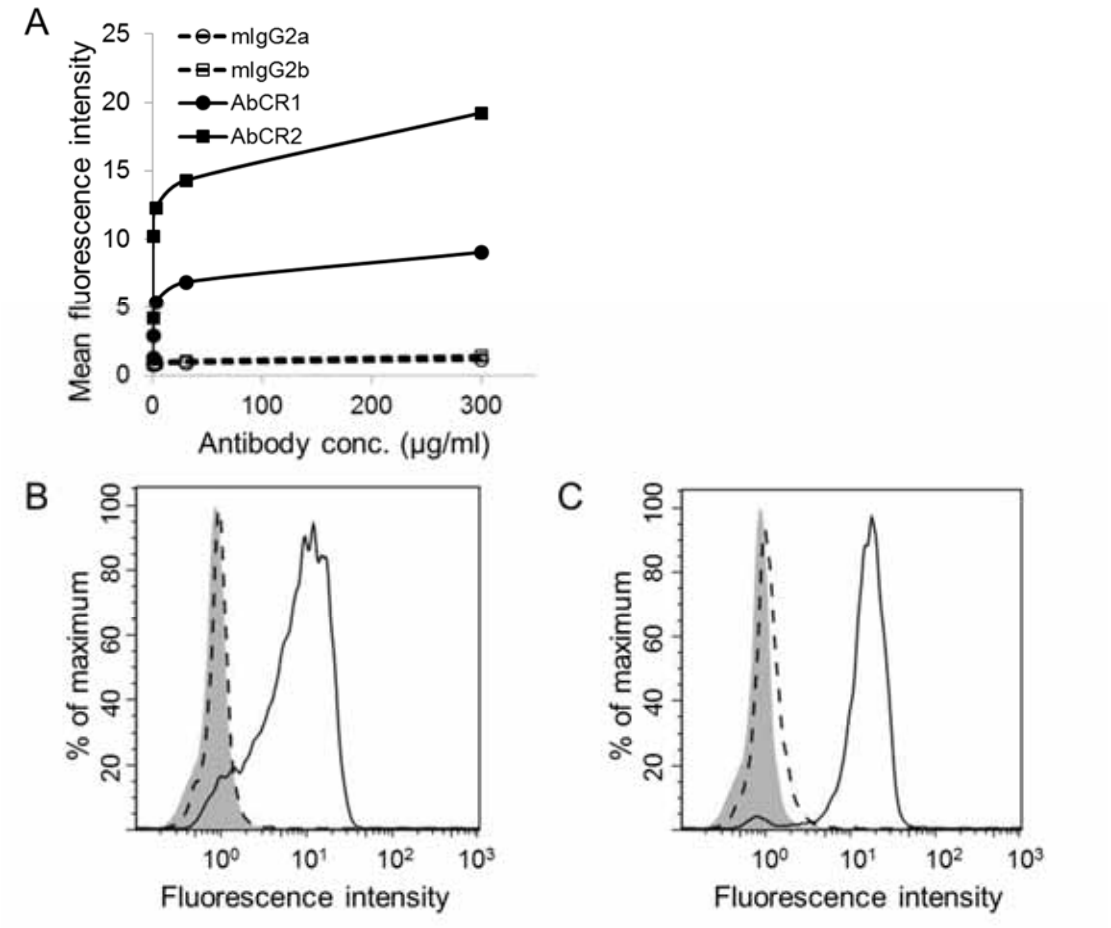

Fig. 3-1 Binding of anti-gpCXCR1 and -gpCXCR2 antibodies to guinea pig neutrophils.

A) Binding of anti-gpCXCR1 antibody, AbCR1 (filled circle with solid line), anti-gpCXCR2 antibody, AbCR2 (filled square with solid line), mouse IgG2a isotype control (open circle with dashed line), and mouse IgG2b isotype control (open square with dashed line) at the indicated concentrations to guinea pig neutrophils with SEM (n=3). Representative results of binding of **B)** 30 μg/ml AbCR1 (solid line), mouse IgG2a isotype control (dashed line) and no antibody (gray filled) and **C)** 30 μg/ml AbCR2 (solid line), mouse IgG2b isotype control (dashed line) and no antibody (grey filled) each at 30 μg/ml.

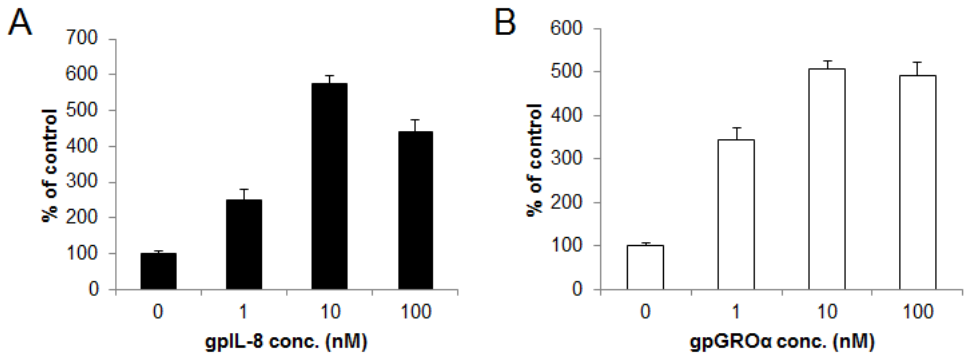

Fig. 3-2 Migration of guinea pig neutrophils induced by gpIL-8 and gpGROα.

Migration of guinea pig neutrophils induced by **A)** gpIL-8 (filled bar) and **B)** gpGROα (open bar). The cells were cultured for 90 min in a Transwell®. The migrated neutrophils in the lower well were counted by flow cytometry. The level of spontaneous migration of cells without chemokine induction was set to 100% as a control. The data are presented as the percent of migration relative to the control with SEM (n=3).

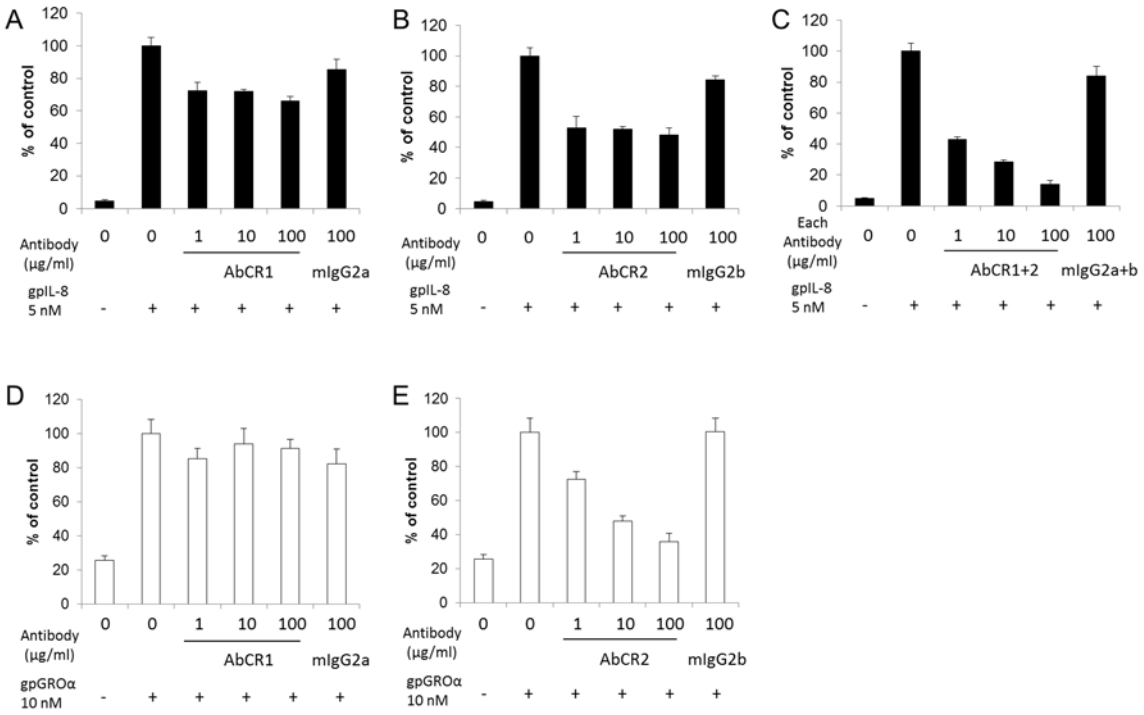

Fig. 3-3 Inhibitory activity of anti-gpCXCR1 and -gpCXCR2 antibodies against migration of guinea pig neutrophils.

Migration of guinea pig neutrophils was induced by 5 nM gpIL-8 (closed bar) in the presence of **A)** AbCR1 or mouse IgG2a isotype control, **B)** AbCR2 or mouse IgG2b isotype control, and **C)** the combination of AbCR1 and AbCR2 or the combination of mouse IgG2a and IgG2b isotype control at the indicated concentrations. Migration of guinea pig neutrophils was induced by 10 nM gpGROα (open bar) in the presence of **D)** AbCR1 or mouse IgG2a isotype control or **E)** AbCR2 or mouse IgG2b isotype control at the indicated concentrations. The migration of cells induced by chemokine in the absence of antibody was set to 100% as a control. The data are presented as the percent of migration relative to the control with SEM (n=3).

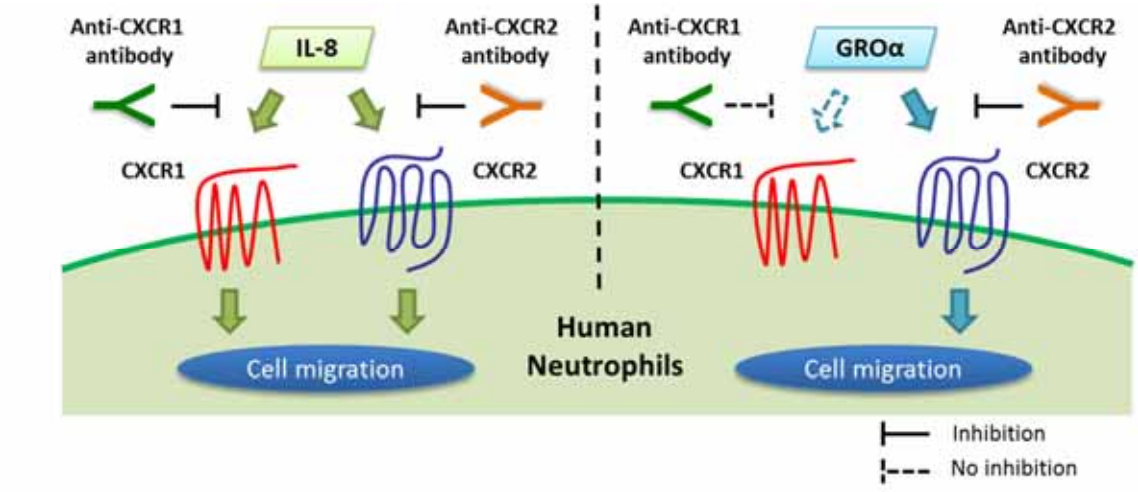

Fig. 3-4 Overview figure of the distinct roles of guinea pig CXCR1 and CXCR2 in neutrophil migration elucidated by inhibitory antibodies.

Chapter 4. Conclusions and future works

4.1 Conclusions of chapter 2

In chapter 2, inhibitory antibodies against guinea pig CXCR1 and CXCR2 were generated. To generate these antibodies against GPCRs, three DNA immunization methods were compared and it was demonstrated that the intramuscular injection of expression plasmid followed by *in vivo* electroporation induced immune response against guinea pig CXCR2 the most efficiently. By this DNA immunization method, monoclonal antibodies against guinea pig CXCR1 and CXCR2 were generated. CXCR1- and CXCR2- dependent migration systems induced by IL-8 and GROα were also established. GpGROα was first produced, and gpIL-8 and gpGROα were proved to have the same receptor selectivity as their human ortholog. In the migration system applied here, anti-gpCXCR1 and -gpCXCR2 specific antibodies showed specific inhibitory activity.

4.2 Conclusions of chapter 3

In chapter 3, the distinct roles of guinea pig CXCR1 and CXCR2 in neutrophil migration were elucidated by using antibodies that inhibit these receptors. First, the expression of CXCR1 and CXCR2 proteins was observed on guinea pig neutrophils by

using generated inhibitory antibodies. These findings revealed that both guinea pig IL-8 and GROα induced dose-dependent migration of guinea pig neutrophils. In this migration system, the use of antibodies against CXCR1 and CXCR2 revealed that both CXCR1 and CXCR2 mediated the migration induced by gpIL-8, and gpCXCR2 mediated the migration induced by gpGROα similar to that in humans.

4.3 Conclusions of this thesis

This thesis aimed at elucidation of the distinct functions of guinea pig CXCR1 and CXCR2 in neutrophil migration by generating and using inhibitory antibodies. The inhibitory antibodies against guinea pig CXCR1 and CXCR2 were generated successfully by DNA immunization, and enabled elucidation of the distinct roles of guinea pig CXCR1 and CXCR2 in neutrophil migration toward IL-8 and GROα. The similarity between human and guinea pig with respect to the distinct roles of CXCR1 and CXCR2 that were demonstrated in this thesis indicated the value of the guinea pig as a model of neutrophilic inflammatory disease.

4.4 Future work

The anti-guinea pig CXCR1 and gpCXCR2 antibodies generated in this work were

proved for the first time to be able to reveal the distinct roles of guinea pig CXCR1 and CXCR2 under physiological conditions. By analyzing the effects of these inhibitory antibodies in an *in vivo* guinea pig model, a deeper understanding of the distinct functions of CXCR1 and CXCR2 should be obtained, particularly with regard to the following two themes.

First, the importance of CXCR1 and CXCR2 inhibition in the guinea pig inflammatory model should be examined for the development of the drugs for inflammatory diseases such as chronic obstructive diseases. A number of CXCR2-specific and CXCR1/2 dual inhibitors for humans were previously studied, but, specific functions of these receptors were not evaluated because of the absence of specific inhibitors in an *in vivo* guinea pig model; the importance of CXCR1 in particular has been elusive. In clinical study, SCH-527123 showed the improvement of lung function was observed in phase II, but a reduction in blood neutrophil count was also observed and the further study was discontinued.[45)] There is a possibility that CXCR1 inhibition is better than CXCR1/2 dual inhibition from the viewpoint of the difference of the distinct roles of CXCR1 and CXCR2. By blocking the activity of CXCR1 and CXCR2 in a guinea pig model, the importance of each receptor can be clarified.

Second, these newly established antibodies should enable us to obtain a deeper understanding of the mechanism of host defense by neutrophils in each tissue. The expression of CXCR1 and CXCR2 on neutrophils in inflamed tissue after their recruitment there differs from that in blood, and it is suggested that this difference leads to the distinct physiological roles of CXCR1 and CXCR2.[46,47] The inhibitory antibodies developed in this work should shed light on this issue by making it possible to evaluate the expression of CXCR1 and CXCR2 proteins on guinea pig neutrophils.

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