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Ig L-chain Shuffling for Affinity Maturation of Phage Library-derived Human Anti-human MCP-1 Antibody Blocking its Chemotactic Activity

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Monocyte chemotactic protein-1 (MCP-1, CC-chemokine ligand 2; CCL2) is involved in the development of various forms of chronic inflammations. Employing the naive human single-chain Fv displaying phage library, we established seven MCP-1-specific scFvs. The MC8 and MC32 clones exhibited blocking activity for the MCP-1-induced chemotaxis of THP-1 cells, in spite of their monovalency. The analysis of V gene usage showed that all clones bore the identical Vh1 gene, IGHV1-24*01, with variable DJ joining sequences, while their Vl usage was relatively varied, suggesting the preferential contribution of the Vh gene. Based on these findings, to minimize the deteriorative influences on the MCP-1 specificity of MC32, we aimed to achieve the affinity maturation of MC32 using MC32 L-chain shuffling library and select MC32 variants. Most MC32 variants increased their affinity by reducing the k_{off} value with no influence of the antigen specificity. MC32 variants #22 or #56 showed ~15-fold higher affinity than MC32, indicating that the L-chain shuffling library is useful if the Vh is dominantly involved in the determination of the antigen specificity.

Key words: affinity maturation, human antibody, L-chain shuffling, MCP-1, phage display library.

Abbreviations: AP, alkaline-phosphatase; BSA, bovine serum albumin; CDR, complementary determining region; FR, framework; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; HAS, human serum albumin; IC50, 50% inhibitory concentration; IgG, immunoglobulin G; Ig L-chain, immunoglobulin light chain; IPTG, isopropyl-thio-β-D-galactopyranoside; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, poly(ethylene glycol); RU, response unit; scFv, single-chain variable fragment; TU, transforming unit.

Monocyte chemotactic protein-1 (MCP-1) is a member of the C-C chemokine family and a potent chemotactic factor for monocytes, T cells, and NK cells (*1*, *2*). MCP-1 has been implicated in a wide range of acute and chronic inflammatory processes characterized by monocyte infiltration, including asthma, glomerulonephritis, rheumatoid arthritis, atherosclerosis, multiple sclerosis, inflammatory bowel disease and meningitis, as well as in the accumulation of macrophages in the tumour sites (*3–6*). In these conditions, monocyte infiltration may be a key early event in disease progression. Thus, the inhibition of the chemotactic activity of MCP-1 is shown to be a potential intervention point for the therapy of these diseases. In a number of animal models, MCP-1-neutralizing antibodies or biological antagonists showed beneficial activities in reducing the inflammation (*7*, *8*).

Recently, antibody medicines are being developed. From the viewpoint of immunogenicity, human antibodies are suitable for use in therapy. Phage-libraries displaying antibody fragments are attractive tools for obtaining human antibodies (*9*, *10*). We have established human scFv-displaying phage libraries and isolated several scFvs with inhibitory activities against a target molecule, such as human IL-18, IL-6 or FcεRIα (*11–13*).

In this study, we attempted to establish human MCP-1-specific human antibodies with inhibitory activity on the chemotactic activity using human scFv-dispalying phage libraries and subsequently to achieve affinity maturation using an IgL-chain shuffling phage library. We isolated seven scFv clones specific to MCP-1 and found that two clones, MC8 and MC32, showed inhibition in the MCP-1-induced chemotaxis of a human acute monocytic leukaemia cell line, THP-1, *in vitro*. The analysis of V gene usage showed that all clones bore the identical Vh1 gene, IGHV1-24*01, with variable DJ joining sequences, while their Vl usage was relatively varied, suggesting a predominant role of the Vh chain in the MCP-1 specificity of these clones.

This unique result suggested that the L-chain shuffling of MC32 may be an effective strategy for affinity

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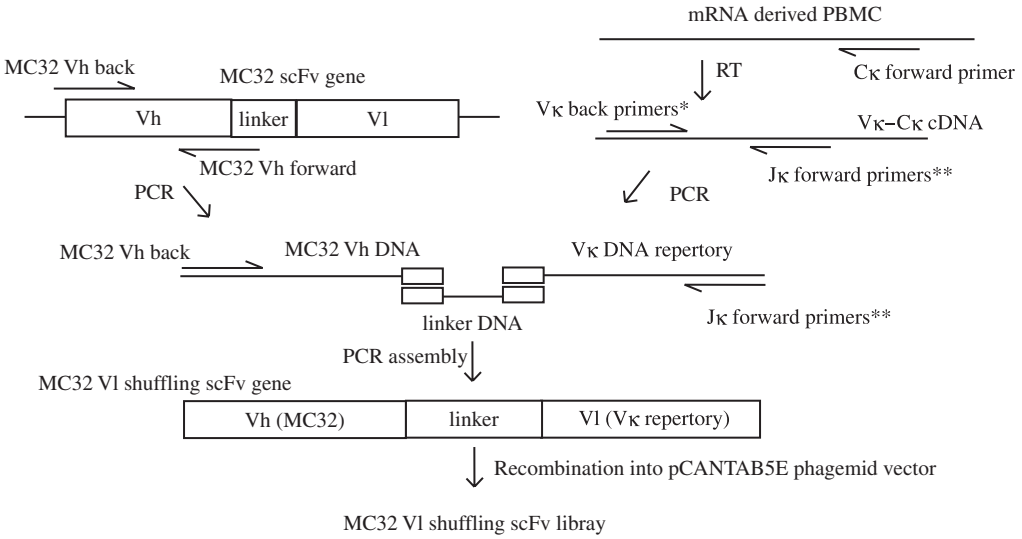

Fig. 1. **Preparation of MC32κ-chain shuffling library.** The Vh gene was amplified from the scFv gene of MC32 by PCR using MC32 Vh back and forward primers. The Vκ genes were amplified from PBMC cDNA using Vκ-specific primers, asterisk: back primers for human Vκ family 1 to 6. double asterisk: forward primers for human Jκ family 1 to 5 (*9*, *15*). The MC32 Vh gene, the Vκ gene repertories and linker DNA encoding $(Gly_4Ser)_3$ were assembled by PCR using these primers to produce a heterogeneous population consisting of varying MC32 Vh-linker-Vκ DNA fragments (MC32 Vκ -shuffling genes). This fragment was inserted into the sites of *Sfi*I and *Not*I of phagemid vector pCANTAB 5E. Phage clones were recovered as described (*11–13*).

maturation, although several other methods have been reported, such as site-directed mutagenesis, error-prone PCR and shuffling of CDRs (*14–18*). We constructed an MC32-L-chain shuffling library, which was prepared by recombining the MC32 Vh chain with a series of κ light-chain repertories in a phagemid vector system. Only after the first round of biopanning with a MCP-1-coated plate, every clone exhibited MCP-1-binding specificity. We examined the K_d value of these MC32 variants and assayed their inhibitory activity on a chemotaxis assay. Seventy-five percent of the variants showed a decreased k_{off} value, resulting in successful affinity maturation in parallel with their IC_{50}. Thus, this study demonstrated that L-chain shuffling approach could minimize the deteriorative influence on the antigen specificity of a parent clone when Vh domains dominantly contribute for its antigen specificity.

MATERIALS AND METHODS

Human Single-Chain Fv-Displaying Phage Library—Four kinds of human scFv-displaying M13 phage libraries were constructed as described previously (*9*, *13*). The V genes for γ, μ, κ or λ chains were separately amplified by PCR. The Vh and Vl gene segments were assembled by PCR with a linker DNA encoding $(Gly_4Ser)_3$ (scFv linkers) to construct a scFv gene. The scFv genes were recombined to the pCANTAB 5E phagemid vector. The scFv display phage was prepared as described (*13*).

L-Chain Shuffling Library—An L-chain shuffling library was prepared according to Marks *et al*. (Fig. 1; *9*, *15*). The Vκ genes were amplified from cDNA that had been prepared for scFv-displaing phage library as described (*13*). In this PCR, six kinds of back primers for human Vκ family and five kinds of Jκ forward primers were employed (*9*, *15*). The Vh gene of MC32 was assembled with these Vκ gene segments together with linker DNA encoding $(Gly_4Ser)_3$ (scFv linkers) by PCR. Vl shuffling scFv gene fragments were re-amplified by PCR using primers containing restriction sites (*Sfi*I and *Not*I). The scFv genes were digested by restriction enzymes (*Sfi*I and *Not*I), gel-purified and ligated to the restriction-digested phagemid vector pCANTAB 5E using T4 DNA ligase.

The diversity of each library was defined as the number of recombinant single ampicillin-resistant clones obtained from consecutive ligation and transformation of the plasmid into competent cells prior to any amplification process of the genes. These libraries contained 6.4×10^6(Vκ 1), 9.8×10^6(Vκ 2), 4.8×10^6 (Vκ 3), 7.2×10^6(Vκ 4), 8.7×10^6(Vκ 5) and 6.4×10^6 (Vκ 6) independent clones.

Biopanning—Biopanning was performed as described by Gejima *et al*. (*12*). Briefly, 5 μg/ml of recombinant human MCP-1 (R&D Systems, Inc., Minneapolis, MN) in 0.1 M $NaHCO_3$ (pH 8.6) was incubated at 4°C overnight in a 35 mm Petri dish (Iwaki Glass, Tokyo). The plates were blocked with 0.5% gelatin, 5% skimmed milk or 1% BSA. A library [5×10^{11} transforming units (TU)] consisted of Vh-Vλ (λ-library), or Vh-Vκ (κ-library) was incubated in an MCP-1-coated plastic plate at room temperature for 1 h. The plates were washed 10 times with PBS containing 0.1% Tween-20 (PBST). The bound phages were eluted with 1 ml 0.1 M glycine-HCl (pH 2.2), immediately neutralized with 0.1 vol of 1 M-Tris–HCl (pH 9.1), and amplified by infection with log-phase *Escherichia coli*, suppressor strain TG1 cells (*sup* E).

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To select more specific phage clones, the amplified eluate was incubated with a decreasing amount of MCP-1 (3 to 1 μg) for subsequent rounds.

Soluble scFv—The soluble scFv was prepared by infecting phage clones with *E. coli* HB2151 (a non-suppressor strain) as described (*11*). These periplasmic scFvs were purified with an anti-E-tag affinity column (GE Healthcare Bio-science Corp, Piscataway, NJ). To eliminate endotoxins, a sample was applied onto Acticlean Etox (Sterogene Bioseparations, Inc., Carlsbad, CA). Gel permeation chromatography was performed as follows: scFvs were loaded on a 10/300 GL Superdex75 (GE Healthcare Bio-science Corp., Piscataway, NJ), which was developed in PBS at a flow rate of 0.4 ml/min. The monomer form or the dimer form of scFv was fractionated.

ELISA—ELISA was performed as described (*19*, *20*). Briefly, ELISA micro titre plates (Nunc, Denmark) were coated with human MCP-1 (80 ng/40 μl/well, R&D Systems, Inc., Minneapolis, MN) or control proteins (80 ng/40 μl/well) overnight at 4°C. The plates were blocked with 1% BSA in PBS (400 μl) at room temperature (RT) for 1 h. Phage clones [40 μl of PEG-precipitated phage (10^{12} TU)] were added to the wells for 1 h at RT. To detect the bound phage clones, biotinyilated anti-M13 mAb (1/1,000 dilution, GE Healthcare Bio-science Corp, Piscataway, NJ) as a primary antibody and alkaline-phosphatase (AP)-conjugated streptavidin (1/1,000 dilution, Vector Laboratories, Inc., Burlingame) were used. Absorbance was measured at 405 nm in incubation with 50 μl of a *p*-nitrophenyl phosphate/10% diethanol amine solution by use of a microplate reader. The soluble scFv antibody was detected using anti-E-tag mAb (1/1,000 dilution, GE Healthcare Bio-science Corp, Piscataway, NJ) in combination with an AP-conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA).

Immunoblotting Analysis—The periplasmic fraction of the HB2151 culture was subjected to SDS–PAGE (12.5%) and blotted to a PVDF membrane using a semidry electroblotter. After blocking with 5% skimmed milk, it was detected by horseradish peroxidase (HRP)-conjugated anti-E-tag mAb using ECL reagents (GE Healthcare Bio-science Corp, Piscataway, NJ) on the image analyzer LAS-1000 (Fujifilm, Tokyo). The NEB pre-stained protein marker (New England Biolabs, Beverly, MA) was used as the protein marker.

DNA Sequencing—The nucleotide sequence of the scFv genes was identified using the Dye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, CA) with primer pCANTAB5-S1 (5′-CAACGTGAAAAAATTATTATTCGC-3′) and pCANT AB5-S6 (5′-GTAAATGAATTTTCTGTATGAGG-3′). The amino acid residues of each variable domain were according to Kabat and Wu (*21*).

Surface Plasmon Resonance Analysis—The BIAcore X100 system (BIAcore AB, Uppsala, Sweden) was used. The MCP-1 (100 μl of 50 μg/ml of 10 mM sodium acetate buffer, pH5.0) was immobilized onto a CM5-sensor chip according to the amine-coupling protocol provided by the manufacturer, and unreacted sites were masked with 1 M ethanolamine-HCl (pH 8.5). The association reaction was initiated by injecting varying concentrations of an scFv solution. The analyte injection was performed at a flow rate of 30 μl/min. The dissociation reaction was performed washing with PBS. At the end of the cycle, the sensor chip surface was regenerated with a 0.1 M glycine-HCl buffer (pH 2.5). The association (k_{on}, $M^{-1}s^{-1}$) and dissociation (k_{off}, s^{-1}) constants were calculated using BIAcore system software (BIAcore X100 Evaluation software). In the case of whole IgG antibody, K_d was calculated using k_{on1} and k_{off1} values by bivalent analyte model whereas that of scFv was evaluated by 1:1 binding model.

Chemotaxis Assay—The migration of cells was assessed in a 48-well microchamber (Neuroprobe, Cabin John, MD) as previously described (*22*). Briefly, the lower wells were filled with 25.5 μl MCP-1 at a concentration of 2.2 nM with or without varying concentrations of scFvs, while the upper wells were filled with THP-1 cells (human acute monocytic leukaemia, 3.75×10^5 cells/50 μl/well). The two compartments were separated by polycarbonate filter with 8 μm pores. After 120 min incubation at 37°C in humidified air-5% CO_2, the filters were removed. The filter was then wiped to remove non-migrated cells on its upside, fixed in paraformaldehyde and stained with Wright–Giemsa stain (Diff-Quik, Sysmex International Reagents Co., Ltd., Hyogo, Japan). The number of migrated monocytes was determined microscopically, and data were calculated as the percentage of the control response induced with MCP-1.

Construction of the MC32 IgG—IgG form of MC32 was constructed as described (*11*). Briefly, the Vh and Vl genes of MC32 were cloned into mammalian cell expression vector pCAG-H with a human IgG1 constant region and pCAG-L with human Cκ, respectively. CHO cells were co-transfected with an equipped molar mixture of these vectors by Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h of culture in a selective medium, the supernatant was harvested from the culture by centrifuge and filtered through a 0.22 μm membrane. IgG was purified using a protein A affinity column. The purified MC32-IgG was analysed by SDS–PAGE.

RESULTS

Establishment of MCP-1-Specific scFvs—The scFv phage clones were selected from scFv phage libraries by three rounds of panning with human MCP-1. We screened 144 clones and found three MCP-1-specific clones from the κ-library (2%); on the other hand, we isolated four MCP-1-specific clones (4.4%) from the λ-library by screening a total of 90 clones. The clones from the κ-library were designated as MC8, MC32 or MC62, and those from the λ-library, as ap20, ap68, apM2 or apM9.

The soluble scFvs were purified from the periplasmic extract of *E. coli* HB2151 on an anti-E tag affinity column. Consisting with the binding specificity of phage clones, all of these scFvs specifically bound to MCP-1 but not to unrelated proteins, including MIP-1α (Fig. 2). Immunoblotting analysis using HRP-conjugated anti-E-tag mAb gave a single protein band corresponding to the expected molecular weight (Fig. 3a). Gel permeation

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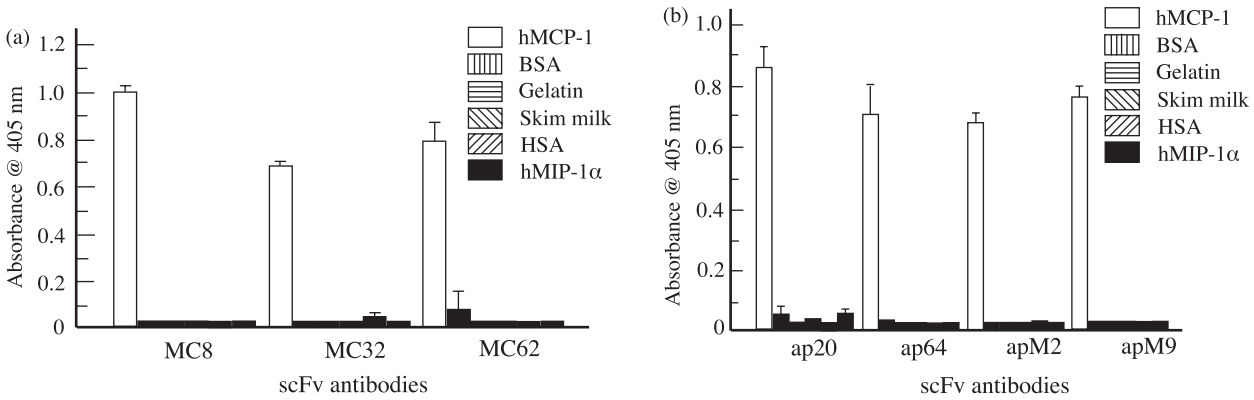

Fig. 2. **MCP-1-specific scFv clones.** ELISA plates were coated with 80 ng/well of MCP-1 and various unrelated proteins for 12 h. Each scFv was tested for its binding specificity as described in MATERIALS AND METHODS section. (a): isolates from the κ-library; (b): isolates from the λ-library.

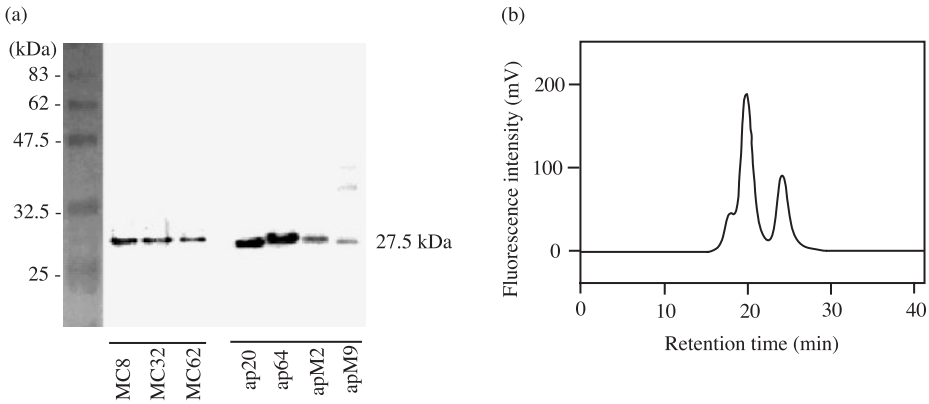

Fig. 3. **Biochemical characterization of scFvs.** (a) Immunoblotting analysis: the periplasmic extract (MC8, MC32, MC62, ap20, ap64, apM2 or apM9) of the HB2151 cell culture was subjected to SDS–PAGE (12.5%). Immunoblotting was performed with HRP-conjugated anti-E-tag mAb. (b) Gel permeation chromatography (10/300 GL Superdex75) of MC32 scFv.

chromatography showed that each preparation of scFvs contained significant amounts of a dimer form in addition to a monomer form (Fig. 3b). Therefore, the HPLC-purified monomer form was freshly prepared and analysed to examine the affinity of scFv.

Nucleotide Sequencing of the scFv Gene—The scFv gene sequences of the selected phage clones were examined (Fig. 4a and b). The germ-line Vh and Vl gene was assigned by sequence homology based on the database (VBASE) of germ-line V-genes compiled by Tomlinson *et al*. (Fig. 4c; *23*). This analysis revealed that all of the isolated scFvs were characterized by germline genes IGHV1-24*01 belonging to the Vh1 gene family. D and Jh are relatively variable. Considering that these clones exhibited equal binding specificity to MCP-1, the similarity of CDR2 among these clones is noteworthy. On the other hand, it is unlikely that CDR3 is preferentially involved in determining the MCP-1 specificity in these scFvs. It is also noteworthy that MC8 and MC32 are different only at one amino acid residue (Q in MC8, while P in MC32 at position 114 of Vh). In contrast, the Vl gene usage appeared to be variable (IGKV1-39∗01 for two clones, IGLV3-21*01 for two clones) while the Jl gene was relatively restricted for usage. These results suggested a dominant contribution of the IGHV1-24∗01 heavy-chain gene, particularly, the Vh-CDR2 region, rather than of the light-chain gene, to the MCP-1-specific-binding activity.

Inhibitory Activity of MCP-1-Specific Human Antibodies on MCP-1-Induced Chemotaxis—To examine the effects of scFvs on the chemotactic activity of MCP-1, we assayed the migration of THP-1 cells using 2.2 nM MCP-1 in the presence or absence of varying concentrations of these scFvs. Since scFvs were prepared from the periplasm of *E. coli*, the contamination of endotoxins may affect the chemotaxis activity of MCP-1. To exclude this possibility, the endotoxin level of the scFv samples was estimated to be below 30 ng/μg-scFv. It was shown that, at this level, endotoxins did not affect THP-1 chemotaxis by MCP-1 (data not shown).

As shown in Fig. 5a and b, MC8 and MC32 scFv clones exhibited the antagonistic activity in a dose-dependent manner. ap20 and ap64 showed weak inhibitory activity at 3.5 μM while apM2 and apM9 showed no inhibitory activity (Fig. 5c).

We constructed the MC32 IgG form using MC32 scFv DNA as described in MATERIALS AND METHODS section. ELISA showed that MC32 IgG preserved its binding

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(a) Amino acid sequences of the Vh domains

```
                    FR1                CDR1      FR2              CDR2
MC8   QVQLQQSGAEVKKPGSSVKVSCKASGGTFS SYAIS WVRQAPGQGLEWMG GFDPEDGETIYAQKFQG
MC32  QVQLQQSGAEVKKPGSSVKVSCKASGGTFS SYAIS WVRQAPGQGLEWMG GFDPEDGETIYAQKFQG
MC62   not determined
ap20  QVQLVQSGAEVKKSGASVKVSCKVSGHTLT DLSMH WVRQAPGAGLEWMG GFDAEEGGAVYAQKFQG
ap64  EVQLVESGAEVKKPGASVKVSCKVSGYTLT ELSMH WVRQAPGKGLEWMG GFDPVDGETIYAQKFQG
apM2  EVQLVESAAEVKKPGESLKVSCKVSGYTLT ELSMH WVRQAPGKGLEWMG GFDPEDGETIYAQKFQG
apM9  QVQLVQSGAEVKKPGSSVKVSCKVSGHTLT ELSLH WVRQAPGKGLEWMG GFDPEDGETIYAQKFQG

                    FR3                CDR3       FR4
MC8   RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT DLGGGDYYYGMDV WGQGTTVTVSS
MC32  RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT DLGGGDYYYGMDV WGPGTTVTVSS
MC62   not determined
ap20  RVTMTEDTSTDTAYLELSSLRPEDTALNYCAS DLSSS-WYDIFDI WGQGSLVTVSS
ap64  RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT DGFS-----GYDS WGQGTLVTVSS
apM2  RVTMTEDTSTDTAYMELSSLRSEDTPVYYCAT AERG--YSYGLDY WGQGTLVTVSS
apM9  RVTVTEDTSTDTAYMELSSLRSEDTAVYYCAT ADTTWDGYYAFDV WGQGTLVTVS-
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(b) Amino acid sequences of the Vl domains

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                    FR1                CDR1          FR2          CDR2
MC8   DIQLTQSPSTLSASVGDRATISC RSSQ--SINTY---LH WYQQKPGEAPKLLIY AASTLQS
MC32  DIQLTQSPSTLSASVGDRATISC RSSQ--SINTY---LH WYQQKPGEAPKLLIY AASTLQS
MC62  DIVMTQSPLSLPVTLGQPASISC RSSQSLVYSDGNTYLN WFQQRPGQSPRRLIY KVSNRDS
ap20  QPVLTQPP-SVSVAPGKTATITC ERSN--IGRKS---VH WYQQKPGQAPVLVIT NDNGRPS
ap64  SSELTQDP-AVSVVLGQTVRITC QGDS--LRSYY---AT WYQQKPGQAPVLVIY GKNNRPS
apM2  SYVLTQPP-SVSVVPGETASISC RGDN--IGSKD---VQ WYQQKPGQAPVLVIY DDEVRPS
apM9  QSVLTQPP-SASGTPGQRVTISC SGSNSNIGSNT---VN WYQQLPGTAPKLLIY SNNQRPS

                    FR3                CDR3       FR4
MC8   GVPSRFSGSGSGTDFTLTITTLQPEDFATYYC QQS---FTTPLT FGGGTKVEIKR
MC32  GVPSRFSGSGSGTDFTLTITTLQPEDFATYYC QQS---FTTPLT FGGGTKVEIKR
MC62  GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC TQS---IQLPLT FGGGTKVEIKR
ap20  GIPERFSGSNSGNAATLTISRVEAGDEADYYC QVWDYSSDHRVQ FGGGTQLTVLG
ap64  GIPDRFSSSTSGNTASLTITGAQAEDEADYYC NSRDSSGNH-VL FGGGTKLTVLG
apM2  GTPERFSGSNSGNTATLTISGVEAGDEADYYC QVWDSSSDH-YV FGTGTQLTVLG
apM9  GVPDRFSGSKSGTSASLAISGLQSEDEADYYC AAWDDRMTGTYV FGTGTKLTVLG
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(c) Variable gene usage

	Heavy chain.			Light chain.	
clone No.	**V**	**D**	**J**	**V**	**J**
MC8	IGHV1-24*01	IGHD3-16*01	IGHJ6*02	IGKV1-39*01	IGKJ4*01
MC32	IGHV1-24*01	IGHD3-16*01	IGHJ6*02	IGKV1-39*01	IGKJ4*01
MC62	N.D.	N.D.	N.D.	IGKV2-30*01	IGKJ4*01
ap20	IGHV1-24*01	IGHD6-13*01	IGHJ3*02	IGKV3-21*01	IGKJ3*01
ap64	IGHV1-24*01	IGHD1-14*01/inv	IGHJ5*02	IGKV3-19*01	IGKJ3*01
apM2	IGHV1-24*01	IGHD5-18*01	IGHJ4*02	IGKV3-21*01	IGKJ1*01
apM9	IGHV1-24*01	IGHD5-24*01	IGHJ3*01	IGKV1-44*01	IGKJ1*01

Fig. 4. **Amino acid sequences and gene usage of MCP-1-specific scFvs.** Amino acid sequences of the Vh (a) and Vl domains (b): the complementary- determining regions (CDR1–CDR3) and the flanking regions (FR1-4) were deduced according to Kabat and Wu (*21*). (c) The germ-line gene usage was assigned based on homology to a database (VBASE) of germline V-genes compiled by Tomlinson *et al*. (*23*).

activity against MCP-1. In the chemotaxis assay, MC32 IgG inhibited the MCP-1-induced THP-1-migration 2.5-fold more strongly than MC32 scFv in reference to IC_{50} (Fig. 5d).

SPR Analysis of MC32 scFv and IgG Form—The affinity of the monomer form of MC32 scFv and IgG was analysed. SPR profiles of MC32 scFv and IgG form were presented in Fig. 6a and b. They showed clear responses in dose–response manners. The K_d of MC32 IgG was almost similar to that of scFv form (Fig. 6c).

Vl Chain Contribution to the Affinity and Anti-MCP-1 Chemotactic Activity of MC32 scFv—The analysis of the V gene usage of MCP-1-specific scFvs suggested its preferential contribution of Vh for the commitment of antigen specificity (Fig. 4). To avoid a strong influence on the MCP-1-binding specificity but acquire an increase of affinity, L-chain shuffling seemed to be one of the choices for affinity maturation. Therefore, we attempted to evaluate the role of the Vl gene by using an MC32 L-chain shuffling library. In this library, the whole repertoire of Vκ genes derived from human PBMCs was

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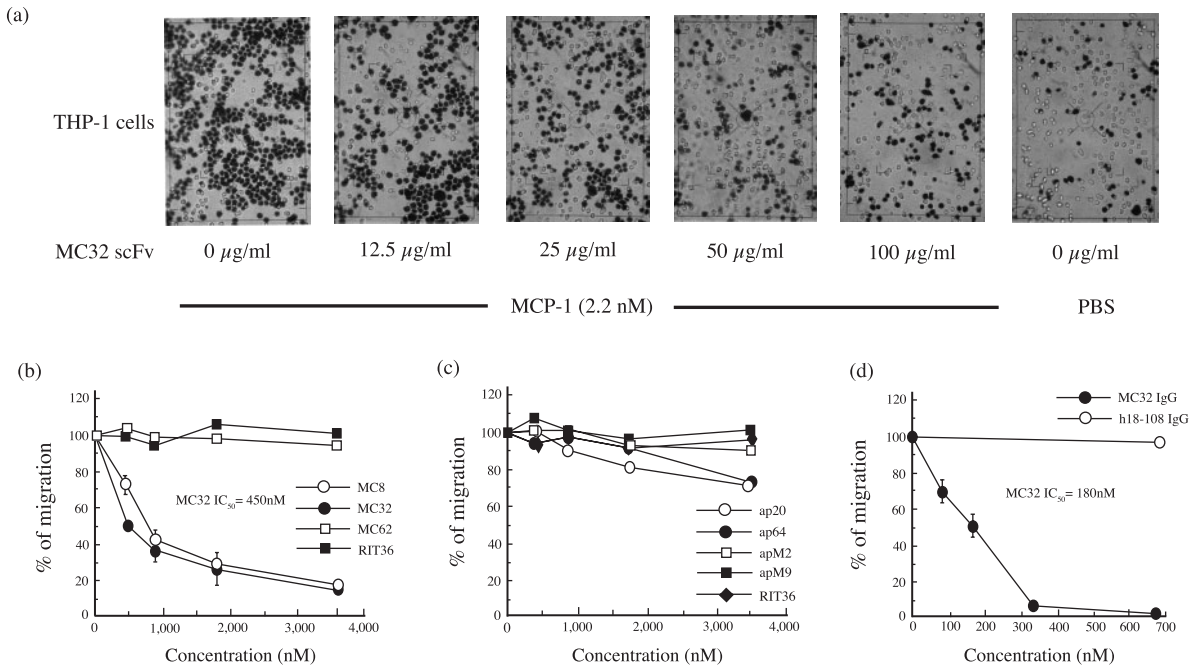
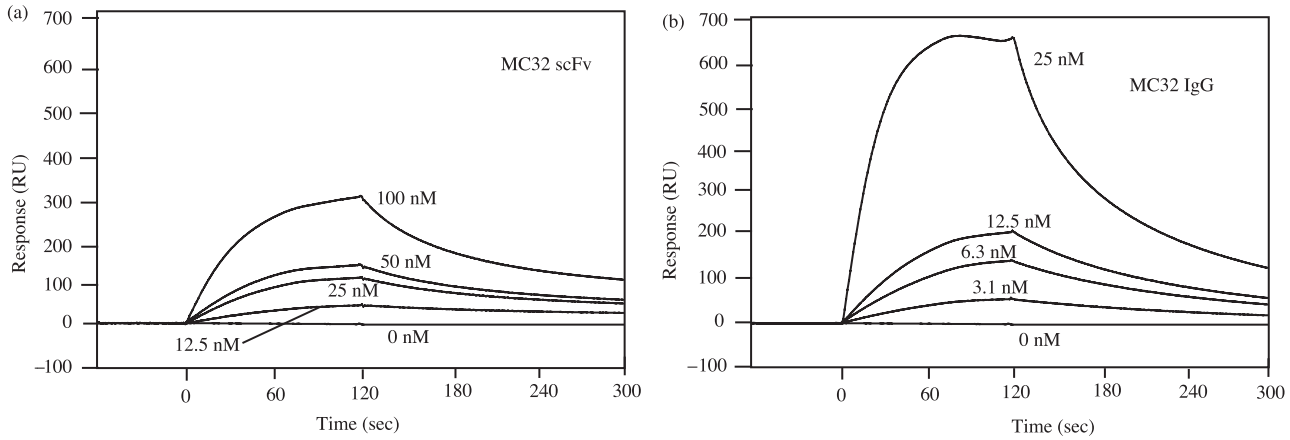


Fig. 5. **Inhibitory activity of MC32 on the MCP-1-induced chemotaxis of THP-1 cells.** (a) Microscopic observation: cells were stained with Wright–Giemsa stain (Diff-Qick). Migration inhibitory activity of MC32 scFv: $IC_{50} = 450$ nM (b), other MCP-1-specific scFvs (c) and MC32 IgG: $IC_{50} = 180$ nM (d): Percentage of migration = [(the number of migrating cells in the experimental group) − (the number of migrating cells in the medium)] ÷ [(the number of migrating cells in MCP-1 with no inhibitors) − (the number of migrating cells in the medium)] × 100. RIT36 is the scFv from an unrelated clone.



(c)

clone No.	k_{on} ($sec^{-1}M^{-1}$)	k_{off} (sec^{-1})	K_d (k_{off}/k_{on})(M)
MC32 scFv	9.3×10^5	2.0×10^{-2}	2.1×10^{-8}
MC32 IgG	6.5×10^5	1.1×10^{-2}	1.7×10^{-8}

Fig. 6. **SPR analysis of MC32 scFv and its IgG form.** (a, b) BIAcore sensorgrams recording the association and dissociation of MC32. The monomer form of scFv or IgG was examined. (c) Summary of k_{on}, k_{off} and K_d. K_d of IgG was calculated using k_{on1} and k_{off1} values by bivalent analyte model whereas that of scFv was evaluated by 1:1 binding model.

recombined with the MC32 Vh chain in a phagemid vector system. From this library, MC32 variants were selected by panning with MCP-1. We screened 60 clones after the first round of biopanning. As expected, every clone showed MCP-1-specific binding activity, suggesting the dominant contribution of Vh to MCP-1-binding specificity (Fig. 7a). To examine the effects of Vl shuffling on MC32 scFv variants, their k_{off} value was analysed

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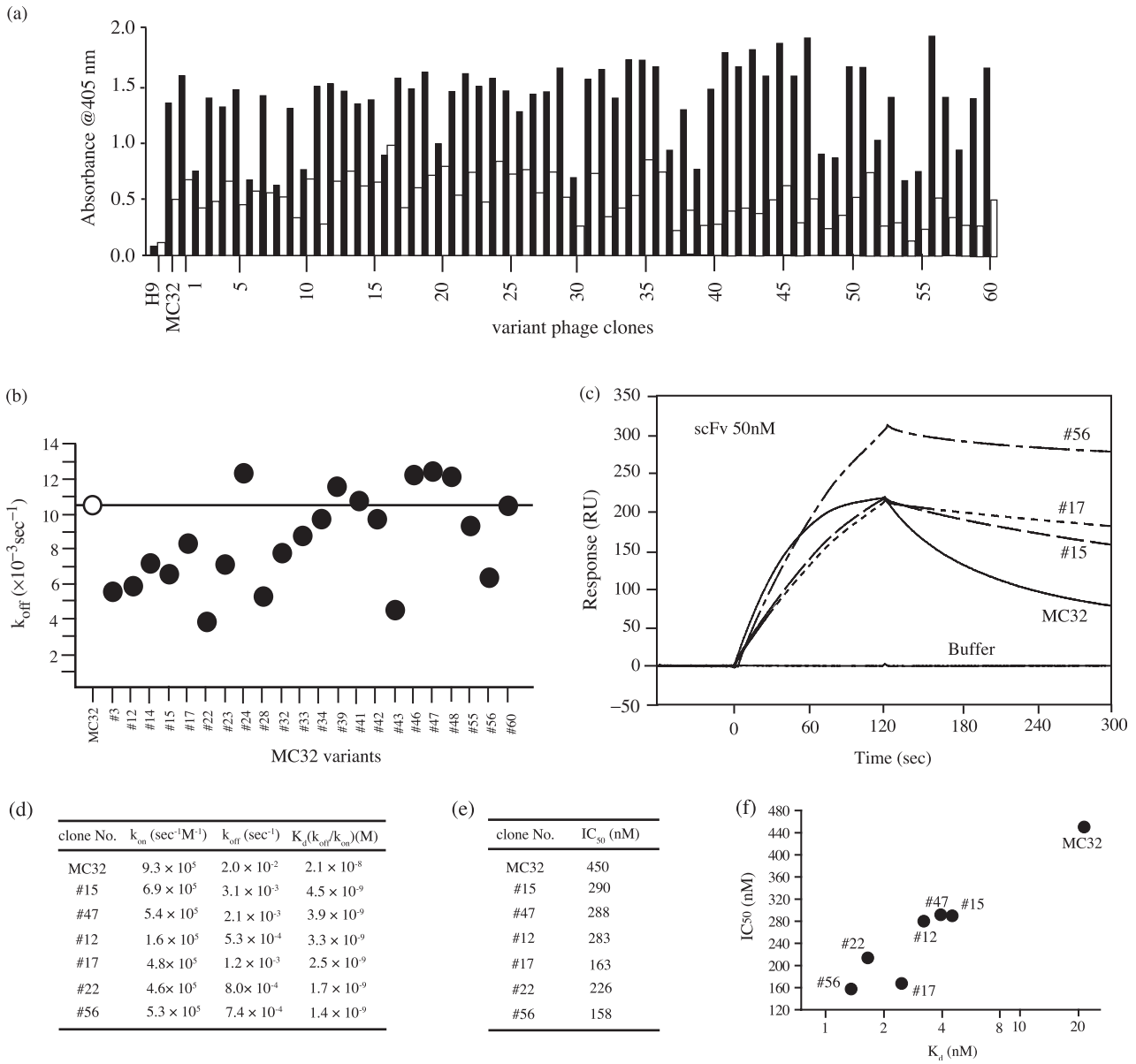

(d)

clone No.	k_{on} ($sec^{-1}M^{-1}$)	k_{off} (sec^{-1})	$K_d(k_{off}/k_{on})$(M)
MC32	9.3×10^5	2.0×10^{-2}	2.1×10^{-8}
#15	6.9×10^5	3.1×10^{-3}	4.5×10^{-9}
#47	5.4×10^5	2.1×10^{-3}	3.9×10^{-9}
#12	1.6×10^5	5.3×10^{-4}	3.3×10^{-9}
#17	4.8×10^5	1.2×10^{-3}	2.5×10^{-9}
#22	4.6×10^5	8.0×10^{-4}	1.7×10^{-9}
#56	5.3×10^5	7.4×10^{-4}	1.4×10^{-9}

(e)

clone No.	IC_{50} (nM)
MC32	450
#15	290
#47	288
#12	283
#17	163
#22	226
#56	158

Fig. 7. **SPR analysis and anti-chemotaxis activity of κ-chain variants of MC32 scFv.** (a) ELISA of κ-chain variants of MC32 selected from the L-chain shuffling library of MC32. ELISA was performed as described in Fig. 2. H9 is the scFv phage from an unrelated clone. (b) k_{off} analysis of MC32 κ-chain variants using crude periplasmic extracts. k_{off} but not k_{on} analysis was possible without information of the secreted scFv concentration. (c) BIAcore sensorgrams recording the association and dissociation of the affinity-purified MC32 κ-chain variants. (d) Summary of k_{on}, k_{off} and K_d of MC32 variants. (d) Summary of the anti-chemotaxis activity of MC32 variants. The chemotaxis assay was performed as described in Fig. 6. IC_{50} was determined from an inhibitory curve. (e) Correlation of the affinity of MC32 variants and their anti-chemotaxis activity. IC_{50} was plotted against the K_d value detected in (d).

using crude scFv preparations with the BIAcore 2000. Seventy-five percent of the variants showed a lower k_{off} values than that of the original MC32 scFv, while 25% of the variants slightly increased the k_{off} (Fig. 7b). When the purified scFvs were examined using the BIAcore X100 to assess their K_d values, the dissociation rates of the variants were slower than that of MC32, while the association rates were similar to that of MC32. Thus, the increase of variant's affinity was mainly affected by reduction of k_{off} value (1- to 15-fold decrease of K_d value).

To assess the relationship of the affinity of scFv to the anti-chemotactic activity of the MC32 variants, we estimated their IC_{50} on the inhibitory activity of MCP-1-induced chemotaxis. As shown in Fig. 7f, the inhibitory activity of these variants was correlated with their affinity.

To investigate the structural basis of these correlations between the affinity and IC_{50} of MC32 variants, their Vl gene sequences were determined (Fig. 8). Except for variant #17, the selected Vls were quite similar. There were a few amino acid substitutions in FR1 but no

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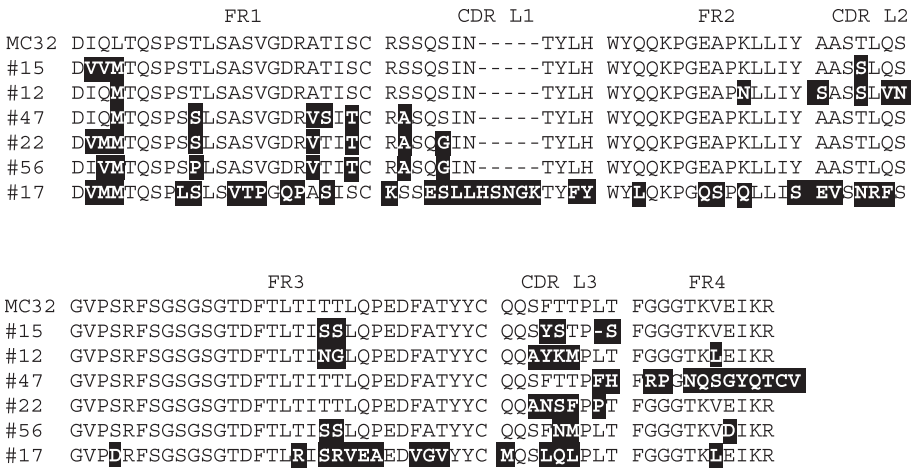

Fig. 8. **Amino acid sequences and gene usage of the MC32 κ-chain variants.** Amino acid sequences of the Vκ domains. The complementary-determining regions (CDR1–CDR3) and the flanking regions (FR1-4) were deduced according to Kabat and Wu (*21*). The different amino acid sequences from that of MC32 are indicated by black boxes.

substitution in FR2 and FR3. The CDR1 and CDR2 of five variants were identical. However, relatively high frequent substitutions were found in CDR3, suggesting that this region influence for decreasing the rates of dissociation of MC32 variants.

DISCUSSION

Employing the naive human scFv phage display libraries, we have isolated seven scFv clones that specifically bound to human MCP-1.

The sequencing analysis revealed that these clones utilized an identical germ line Vh gene, IGHV1-24*01, which belongs to the Vh1 gene family. However, their D and J segments were variable. The light-chain genes, Vl and Jl, were also variable. When the similarity among CDRs of Vh was examined, CDR1 and CDR3 were variable, while CDR2 was relatively similar among seven scFv clones. These results suggested that the CDR2 of Vh might contribute to the determination of MCP-1 specificity. Despite the Vh gene similarity, however, only two clones, MC8 and MC32, blocked the MCP-1-induced chemotaxis of THP-1 cells. These two clones have an identical sequence except for one amino acid residue at position 114 of Vh. A comparison of the Vh sequences of these inhibitory clones to non-inhibitory clones suggests that CDR1 may contribute to the inhibitory activity of these clones because only CDR1 is unique in inhibitory clones. It is of note that these clones inhibited the MCP-1-induced chemotaxis despite their monovalency. The IgG form of MC32 was constructed to attain increases in chemotaxis-inhibitory activity. Divalency of IgG resulted in ~2.5-fold increase of its inhibitory activity although its K_d was almost similar to that of scFv. It is also conceivable that IgG form might gain the stability than scFv.

Affinity maturation of an antibody is generally attained by error-prone PCR in the selected region or chain shuffling (*14–18*). As Vh domains dominantly contribute for MCP-1-specificity (Fig. 4), the L-chain shuffling strategy was undertaken to attain affinity maturation in scFv form. This approach may minimize the deteriorative influences on the MCP-1-binding specificity of MC32. When MCP-1-binding clones were selected from the L-chain shuffling library, every clone exhibited binding specificity to MCP-1, strongly suggesting the dominant contribution of IGHV1-24*01 to MCP-1 specificity.

We isolated 60 MCP-1-binding clones and evaluated the binding feature by SPR analysis. As expected, L-chain shuffling mildly influenced the binding features of MC32, and variable clones were identified. Using purified scFvs, it was shown that #22 and #56 increased their affinity, ~15-fold in comparison with that of MC32. Their IC_{50} of the anti-chemotaxis activity appeared to correlate with the binding affinity of scFv clones although it was not accurately related with an increase of affinity. The structural search of this affinity maturation by sequencing of these Vl genes suggested that CDR3 contribute to this effect. Thus, in this particular case, in which the binding specificity was dominantly determined by Vh or Vl, the counter chain-shuffling library may be effective to achieve affinity-maturated clones without abolishing the given antigen specificity.

Several variants selected from the MC32 VL shuffling library were improved regarding their affinity. In the changes of affinity, k_{off} value was more effective than k_{on} value.

Recently, an MCP-1-neutralizing IgG, CNTO 888, originated from a phage library, HuCAL, has been reported (*24*). MC32 and #56 may also be useful for a variety of chronic inflammatory diseases. The Vh gene engineering of #56 including IgG conversion to attain further affinity maturation remains to be achieved.

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