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Production of Human Monoclonal Antibodies against FcεRIα by a Method Combining *in Vitro* Immunization with Phage Display

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An *in vitro* immunization protocol using human peripheral blood mononuclear cells (PBMC) was developed to generate human antigen-specific antibodies. Monoclonal antibodies have great potential, and in particular, efficient acquirement of monoclonal antibodies against membrane proteins provides advantages. In this study, we tried to generate a human monoclonal antibody against the high affinity IgE receptor, FcεRIα, using a method combining *in vitro* immunization and phage display. Heavy and light chain variable region genes were obtained from PBMC immunized *in vitro* with FcεRIα-expressed KU812F cells. Subsequently a combined phage antibody library 6×10^3 in the size was generated. Antigen-specific phage antibody clones were selected by panning with recombinant FcεRIα and recombined to produce human IgG format antibodies using CHO cells. The antibodies exhibited specific binding against FcεRIα. These results suggest that one can obtain membrane protein-specific human monoclonal antibodies from a relatively small phage antibody library using *in vitro* immunized PBMCs.

Key words: IgE receptor; *in vitro* immunization; human monoclonal antibody; phage display

Monoclonal antibodies (mAbs) are proteins that have very specific, sensitive reactions with specific sites on target molecules. As a result, they have become important tools in modern biological research and have great clinical potential, particularly in the analysis and treatment of human disease. The advent of mAbs was done using hybridoma technology, which was introduced by Köhler and Milstein.[1)] However, owing to their murine origins, the first generation of mAbs evaluated in the clinic was limited by their immunogenicity and limited ability to recruit immune effector mechanisms.[2–4)] Hence, increasing effort has been applied to engineer antibodies without a non-human structure minimizing such responses.[5–7)] To overcome this problem, we developed an *in vitro* immunization (IVI) protocol[8–10)] that elicits antigen-specific immune responses in peripheral blood mononuclear cells (PBMCs). Using this protocol, antigen-specific antibody genes were obtained against soluble antigens, including rice allergen, Cholera toxin B subunit (CTB), mite extract, and TNF-α.[11,12)] In antibody engineering, cell surface antigens are often targeted[13)] since they play an increasingly important role, especially in cancer diagnosis and therapy,[14)] but the generation of antibodies against cell surface antigens is difficult owing to the difficulty in obtaining sufficient amounts of antigen at high purity and in the native conformation for immunization.[15)]

In this study, we attempted to generate antigen-specific monoclonal antibodies against membrane proteins using intact cells as the antigen. We focused on the high affinity IgE receptor epsilon alpha chain (FcεRIα), which triggers allergic reactions[16)] and is expressed on human basophile cell line KU812F. We attempted to obtain FcεRIα-specific human monoclonal antibody using a method combining IVI and phage display.

Materials and Methods

Antigens and reagents. Recombinant human interleukin 2 (IL-2) was purchased from Genzyme (Cambridge, MA). Recombinant human IL-4 was from Pepro Tech (London, UK). L-leucyl-L-leucine methyl ester (LLME) was from Bachem (Torrance, CA). D-type and K-type CpG ODN (5′-ggTGCATCGATGCAGGGGggG-3′, 5′-tcgagcgttctc-3′) (uppercase and lowercase letters indicate bases with phosphodiester and phosphorothioate-modified backbones respectively) were from Sigma Genosys (Hokkaido, Japan).[17)] Fish gelatin (FG) was from BioFX laboratories (Owings Mills, MD).

Recombinant FcεRIα. Total RNA from KU812F cells was prepared with a GenElute Mammalian Total RNA Kit (Sigma-Aldrich, St. Louis, MO) and cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI). The DNA fragment coding FcεRIα was amplified by PCR by using sense primer (5′-CGCGGATCCGCAGTCCCTCAGAAACCTAAGG-3′; underlining indicates the site at which *Bam*H I was added) and anti-sense primer (5′-CCCAAGCTTCGGAGCTTTTATTACAGTAATGTTGAGG-3′; underlining indicates the *Hind* III site). The PCR product was ligated to the pET-29a plasmid, in which codons corresponding to six histidine tags were inserted upstream of the stop codon. The entire sequence was confirmed by sequencing analysis. The FcεRIα expression vector was transfected into *Escherichia coli* (*E. coli*) strain BL-21-DE3. Gene expression was induced with kanamycin including Overnight Express Instant TB medium (Novagen, Darmstadt, Germany). Recombinant FcεRIα (rFcεRIα) was purified from the cell lysate by affinity chromatography using a His trap column (GE Healthcare, Uppsala, Sweden).

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In vitro *immunization.* The preparation of PBMCs and *in vitro* immunization were performed as previously described.[12] LLME-treated PBMCs were sensitized with KU812F cells fixed with 1% glutaraldehyde or a lysate prepared from the same number of KU812F cells, and cultured in eRDF medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, IL-2, IL-4, 2-mercaptoethanol, and D-type CpG ODN (1 mM). After culture for 3 d, K-type CpG ODN (1 mM) was added and the cells were cultured for an additional 3 d.

All the experiments in this study were carried out in accordance with the principles of the Declaration of Helsinki and the regulations of the Ethics Committee of the Faculty of Agriculture of Kyushu University.

Enzyme-linked immunosorbent assay (ELISA). The total amounts of immunoglobulin (IgM and IgG) secreted into the culture supernatant were determined by ELISA, as previously described.[18]

Enzyme-linked immunospot analysis (ELISPOT). B cells producing antigen-specific antibodies were detected by ELISPOT assay, as previously described.[10] *In vitro* immunized PBMCs were cultured for 6 d, transferred to a Multiscreen HA plate (Millipore, Bedford, MA) coated with fixed KU812F cells, and cultured for 48 h. After washing with PBS containing 0.05% Tween 20 (TPBS), the secreted KU812F-specific IgM was reacted with horseradish peroxidase conjugated goat anti-human IgM antibody and detected using True Blue peroxidase substrate (KPL, Gaithersburg, MD).

Generation of phage antibody library. The phage antibody library was constructed as previously described.[12] The genes of the immunoglobulin heavy chain variable region (VH) and light chain variable region (VL) were amplified by PCR using the appropriate family-specific primers.[19,20] The amplified VH and VL genes were connected by the DNA linker encoding $(Gly_4Ser)_3$ in the direction of the 5′-VH-linker-VL-3′, and the recombinant single chain Fv (scFv) fragment was then inserted into phagemid vector pCANTAB5E. The recombinant pCANTAB5E was transfected into *E. coli* TG1 cells. Ampicillin-resistant cells were infected with M13KO7 helper phage and grown. A complete phage particle was then released into the supernatant.

Selection of FcεRIα-specific phage. We used streptavidin magnetic beads (Invitrogen, Carlsbad, CA) to select the antigen-specific phage antibody. Prior to panning, the magnetic beads were blocked with 2% FG for 1 h. After the phage antibodies were incubated with biotinylated rFcεRIα for 1 h at room temperature, the streptavidin magnetic beads were added. After incubation for 1 h, the beads and bound phages were pulled down with the magnet. The beads and bound phages were washed 7 times with 1 ml of TPBS, twice with blocking buffer, and once with PBS. The bound phages were eluted with 0.2 M glycine-HCl (pH 2.2) and used to infect early log phase *E. coli* TG1 cells. After incubation for 1 h at 37 °C, the suspension was spread on SOBAG plates and cultured overnight at 37 °C.

Evaluation of the antigen specificity of the phage antibody. The culture supernatants containing phage antibodies were used for in ELISA to examine their binding affinity to rFcεRIα. Microtiter plates (Nunc, Roskilde, Denmark) were coated with rFcεRIα in carbonate buffer at a concentration of 10 μg/well and incubated overnight at 4 °C. After washing, the plates were blocked with 1% FG in PBS for 2 h at 37 °C. After washing of the plates, 100 μl of the culture supernatants was added to the wells and this was incubated for 2 h at 37 °C. After washing of the plates with TPBS, diluted HRP-conjugated mouse anti-M13 mAb (GE Healthcare, Uppsala, Sweden) was added, and the plates were incubated for 2 h at 37 °C. After washing of the plates with TPBS, we added a substrate solution containing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) at a concentration of 0.3 mg/ml. The absorbance was measured at 405 nm using a microtiter plate reader (Tecan, Männedorf, Switzerland).

Production of recombinant human monoclonal IgG in Chinese hamster ovary cells. We amplified the VH and VL genes by PCR using vectors expressing scFv specific for rFcεRIα as templates and appropriate primers. The VH and VL genes were digested with *Sfi* I and *Xho* I and with *Asc* I and *Kas* I respectively, and cloned into the pSecTag2A/IgGc-bearing heavy chain constant region (CH) gene and the pSecTag2A/IgLc-bearing light chain constant region (CL) gene respectively. Chinese hamster ovary (CHO) cells were transfected with these expression vectors using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The IgG in the supernatant was purified by affinity chromatography with protein G column (GE Healthcare, Uppsala, Sweden).

Evaluation for FcεRIα-specific binding of recombinant human IgG. Competitive ELISA was performed to assess the binding of the antibodies to rFcεRIα. After microtiter plates were coated with rFcεRIα at a concentration of 10 μg/well and incubated at 4 °C overnight, the plates were then blocked with 1% ovalbumin in PBS at 37 °C for 2 h. After washing of the plates, recombinant human IgG was added and the plates were incubated at 37 °C for 2 h with serial dilutions of rFcεRIα, rice allergen, β-lactoglobulin, or BSA. After the plates were washed with TPBS, diluted goat anti-human IgG HRP (Biosource) was added and the plates were incubated at 37 °C for 2 h. After the plates were washed with TPBS, the substrate solution containing ABTS was added. The absorbance was measured at 405 nm using a microtiter plate reader.

Flow cytometric analysis. KU812F cells were incubated with the generated anti-FcεRIα monoclonal IgGs or control human IgG in 100 μl of PBS containing 5% BSA. After incubation for 30 min on ice, the cells were washed with PBS and incubated on ice with FITC-labeled anti human IgG or FITC-labeled human IgE for 30 min. After washing, the cells were suspended in 500 μl of PBS and the fluorescence intensity was measured with a FACSAria™ Cell Sorter (BD Bioscience, San jose, CA).

Results

KU812F-specific immune response elicited by in vitro *immunization*

To activate and induce antigen-specific immune responses, human PBMCs were first treated with LLME. Since peripheral blood lymphocytes are strongly suppressed by immunosuppressive cells, including monocytes, no sensitization to antigens occurs if one uses of PBMCs directly isolated from human blood. LLME treatment removes immunosuppressive cells, including NK cells and monocytes, and enables PBMCs to be immunized with any antigen, including human antigens, in the presence of cytokines *in vitro*. Hence, PBMCs were immunized *in vitro* with fixed KU812F cells in the presence of IL-2 and IL-4, and D-type and K-type CpG ODN. Figure 1A shows that the PBMCs, immunized *in vitro*, augmented the cell-cell interactions and consequently formed numerous clusters larger than the control cells. The PBMC immunized with fixed KU812F produced large amounts of IgM and IgG (data not shown) and KU812F-specific antibodies (Fig. 1B). These data indicate that the *in vitro* immunization method using fixed cell antigen can activate PBMCs and induce cell surface antigen-specific immune responses when sensitized with intact cells just as sensitized with lysate soluble antigen.

Construction of the phage display library and selection of antigen-specific phage antibody by panning

The variable region genes of the antibody were amplified from cDNA prepared from PBMCs immunized *in vitro* by PCR using V region family specific primers. Next we constructed and obtained scFv phage display libraries containing 6.4×10^3 clones from

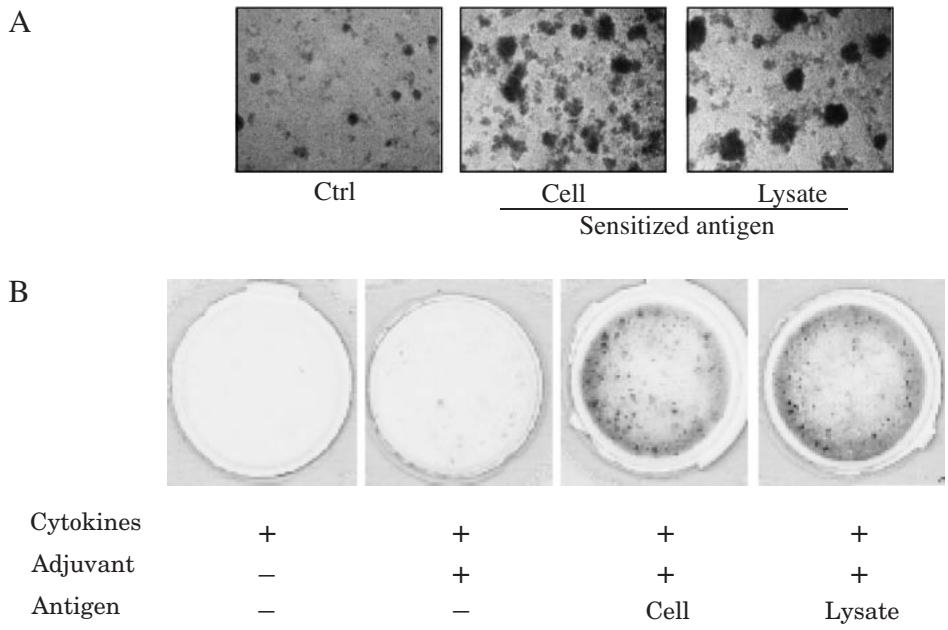

Fig. 1. Immune Responses of Lymphocytes Augmented by *in Vitro* Immunization.
LLME-treated PBMCs were cultured in eRDF medium supplemented with 10% FBS, IL-2, and IL-4 (ctrl), or sensitized with KU812F cells in eRDF medium supplemented with 10% FBS, IL-2, IL-4, and CpG ODN (cell or lysate). A, After culture for 6 d, the PBMCs were observed under a phase contrast microscope. B, KU812F cell-specific IgM derived from PBMCs immunized *in vitro* were evaluated by ELISPOT assay.

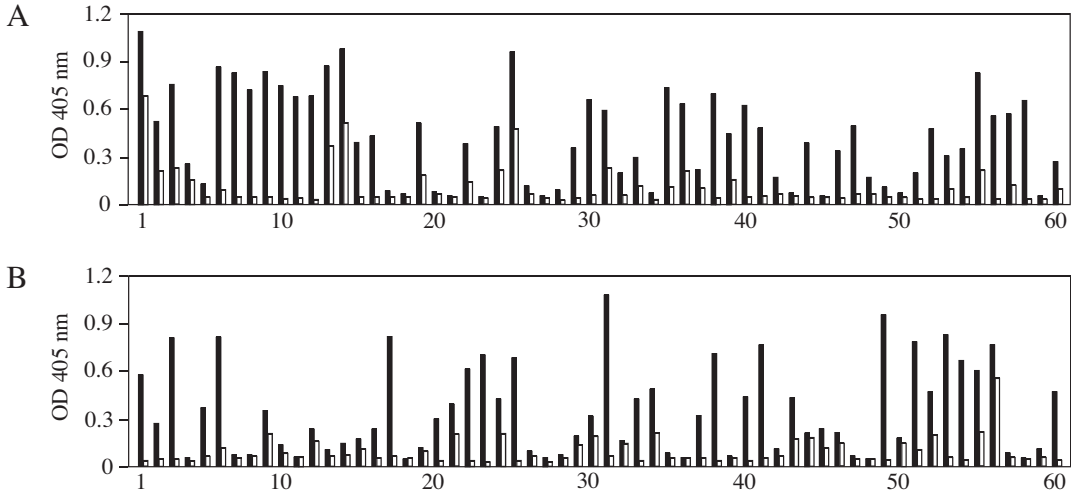

Fig. 2. Selection of Antigen-Specific Phage Antibodies by ELISA.
A, KU812F cell-immunized scFv library. B, KU812F cell lysate-immunized scFv library. The solid and open bars indicate phage antibody binding to rFcεRIα and FG respectively. After panning, 60 individual colonies were selected and infected with M13KO7 helper phage to produce the phage antibodies. The rFcεRIα-specific phage antibody in the culture supernatant was detected by ELISA. FG was used as the non-specific and negative-control antigen.

PBMC immunized with cells and 2.4×10^4 clones from PBMC immunized with lysate. The antigen-specific scFv phages were selected from the phage libraries by panning with streptavidin-magnetic beads[21] conjugated with rFcεRIα in liquid phase. After panning, 60 clones were selected randomly and screened by ELISA for binding ability to rFcεRIα. As Fig. 2 shows the binding activity of monoclonal scFv displayed phage antibodies to rFcεRIα. Several phage antibody clones exhibited specific binding to rFcεRIα. ScFv phages from PBMC sensitized with cell antigen (Fig. 2A) demonstrated higher reactivity for rFcεRIα than from PBMCs sensitized with lysate antigen (Fig. 2B).

Production of the IgG type antibody and functional analysis

The VH and VL genes of the antigen-specific scFv phage antibodies were amplified and cloned into their respective expression vectors. These vectors were transfected into CHO cells and cultured for several weeks. The culture supernatants containing recombinant human IgG were collected, and their antigen specificity was measured by ELISA. As shown in Fig. 3A, three clones, C9, C51, and L49, exhibited specific binding to FcεRIα, but the other clones lost antigen-specific binding. The three FcεRIα-specific individual clones were purified from these culture supernatants of recombinant CHO cells using the MAb trap kit (GE Healthcare) and we

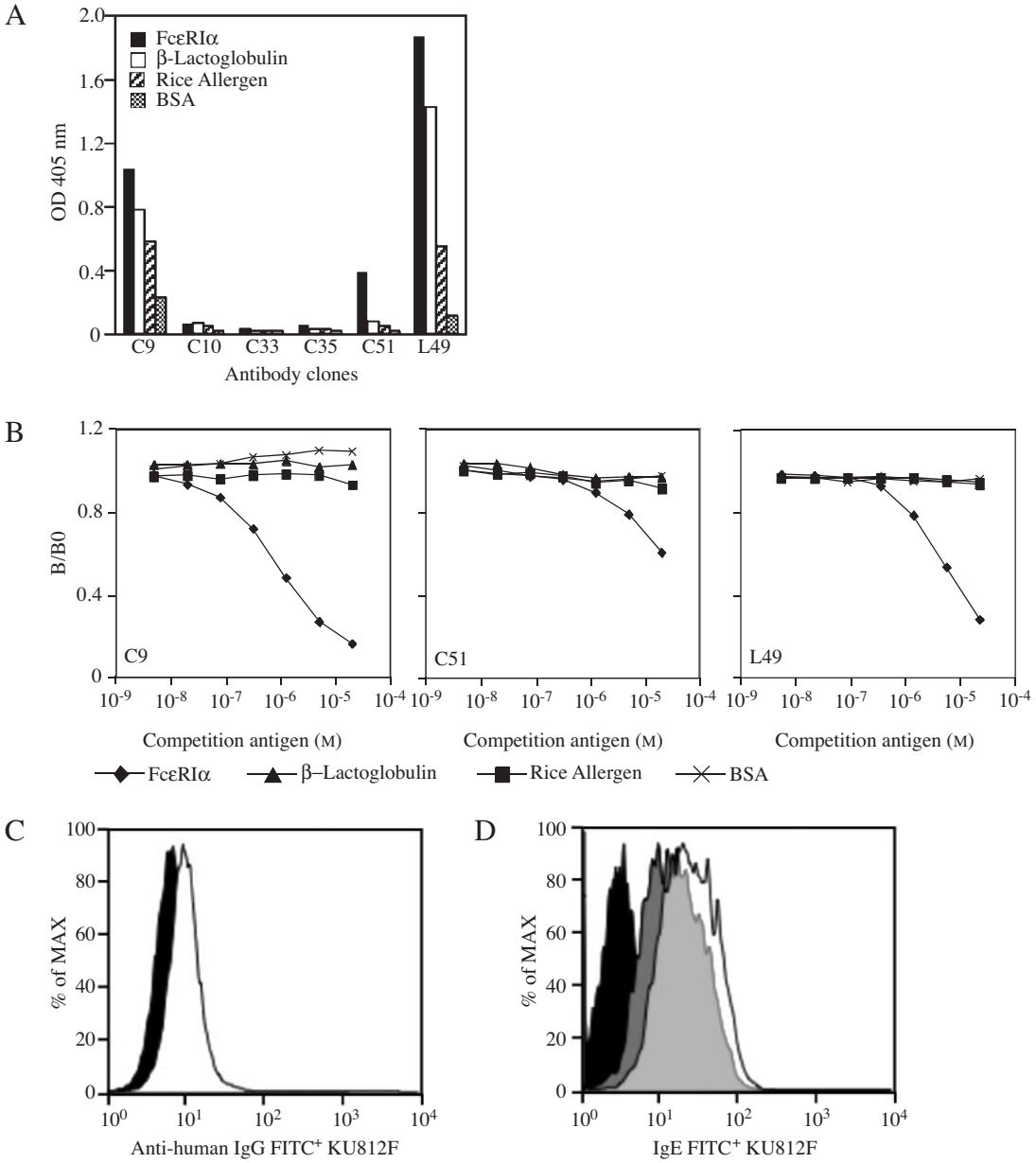

Fig. 3. Analysis of Monoclonal IgG Clones against FcεRIα.

A, Selected scFv phage antibody clones (C9, C10, C33, C35, C51, and L49) were converted to human IgG type and produced by CHO cells. ELISA was used to evaluate the antigen specificity of these antibodies in the culture supernatants. B, A microtiter plate was coated with recombinant FcεRIα. After blocking of the plate, the purified mAbs (C9, C51, and L49) were added with serially diluted competitive antigens. The bound mAbs were captured by goat anti-human IgG-HRP and detected using a substrate solution containing ABTS. B/B0 is the ratio of the absorbance at the last step of ELISA in the presence of various concentrations of a competitive antigen to the absorbance in the absence of the competitive antigen. C, KU812F cells were incubated with mAb C9. MAb C9 was captured with FITC-conjugated anti-human IgG and detected by flow cytometric analysis. Filled peak, binding of human IgG for control. Open peak, binding of mAb C9. D, KU812F cells were treated with FITC-conjugated human IgE after incubation with mAb C9. Inhibition of the IgE binding was detected by flow cytometric analysis. Solid peak, negative control. Open peak, binding with FITC-conjugated IgE. Gray peak, binding with FITC-conjugated IgE after treatment with mAb C9.

performed competitive ELISA to assess the antigen specificity of purified IgGs. We found that all three IgGs bound specifically to rFcεRIα (Fig. 3B). Next we determined whether the generated recombinant FcεRIα specific IgG would recognize FcεRIα expressed on the KU812F cell surface membrane by flow cytometric analysis. C9 exhibited binding ability to FcεRIα expressed on the cell surface of KU812F cells (Fig. 3C), but the other two IgG clones (C51 and L49) did not. Human C9 IgG did not recognize FcεRIα expressed on the cell surface of rat basophil RBL2H3 cell line cells (data not shown), suggesting that this C9 IgG specifically recognizes human FcεRIα.

Next we investigated the ability of C9 anti-FcεRIα IgG to inhibit the binding of IgE to FcεRIα on KU812F. The KU812F cells were preincubated with C9 IgG for 30 min, and then were treated with FITC labeled human IgE. The binding of IgG to FcεRIα expressed on the cells was detected by flow cytometric analysis. As shown in Fig. 3D, C9 IgG inhibited the binding of IgE to FcεRIα on KU812F.

Discussion

Recent studies have demonstrated efficient generation of specific monoclonal antibodies against cell surface

antigens by several methods,[22,23] because efficient antibody production is critical for antibody-based medicine. Here we report a new method of generating human monoclonal antibody against cell surface antigens by combining *in vitro* immunization of human PBMCs with phage display. Fixed KU812F cells elicited antigen-specific antibody production from PBMCs by *in vitro* immunization, as shown in Fig. 1. Initially, we tried *in vitro* immunization of PBMCs with live KU812F cells without fixation, but these PBMCs neither formed clusters nor induced antigen-specific immune responses. Since KU812F cells are anchorage-independent, efficient interactions between KU812F cells and PBMCs can not occur without fixation of KU812F cells. Hence, the fixation process is required to induce immune responses *in vitro*.

The rFcεRIα-specific phage antibody library constructed from cell immunized PBMCs appeared to have better specificity than the library constructed from lysate immunized PBMCs, as shown in Fig. 2. Additionally, in our previous study, we found that VH gene expression in PBMCs immunized *in vitro* was considerably enhanced compared with non-stimulated PBMCs.[12] Furthermore, the affinity of antibodies that can be isolated from naïve libraries is intrinsically linked to the library size. For example, a library containing 3×10^7 clones resulted in antibodies with micromolar affinities.[19] Meanwhile, we obtained antibodies with micromolar affinities from an *in vitro*-immunized library containing 6.4×10^3 clones. These results suggest that an efficient immune response against cell surface membrane protein was induced by *in vitro* immunization with a fixed cell providing the antigen.

We selected and characterized the specificity of IgG antibodies generated against rFcεRIα. First we performed screening of highly reactive IgG clones to rFcεRIα by ELISA using a microtiter plate coated with antigens. Next we selected three clones, C9, C51 and L49, which were examined by competitive ELISA. As shown in Fig. 3A, the antibodies generated showed high reactivities toward rFcεRIα, but cross-reacted with βLG and RA on ELISA with a solid phase of antigens. However, as shown in Fig. 3B, the three clones showed specific binding to rFcεRIα on competition ELISA with a liquid phase of competition antigens. These antibody clones were obtained from the phage display library by panning with recombinant FcεRIα bound to streptavidin magnetic beads in liquid phase. Immobilization of proteins on plastic media often leads to conformational changes in the target protein,[24] which affect antibody specificity. Meanwhile, competitive ELISA can examine the reaction of the antibody in liquid phase. The dissociation constants of the antigen-antibody interactions were determined by competition ELISA with rFcεRIα soluble protein expressed in *E. coli*. C9 IgG inhibited the binding of IgE to FcεRIα on KU812F cells, as shown in Fig. 3C, and the dissociation constant of C9 IgG against intact FcεRIα might be higher than that against rFcεRIα, but it is difficult to determine the dissociation constant against intact FcεRIα expressed on the cell membrane of KU812F cells, because the molar number of FcεRIα molecules expressed on the membrane of KU812F cells is unknown. Taken together, this suggests that the antibody clones bound to soluble antigen and that the affinity receptor, FcεRIα[25,26] on mast cells and basophiles, is central to allergic diseases.[16] When receptor-bound IgE forms cross-links with a multivalent allergen, the resulting activation signal causes the release of inflammatory mediators. The C9 IgG generated bound to FcεRIα expressed on KU812F cells specific and blocked binding between FcεRIα and IgE. This suggests that C9 IgG might inhibit allergic interactions on the molecular level.

In summary, we developed a human monoclonal antibody against a receptor protein using a small phage antibody library and PBMCs immunized *in vitro* with intact whole cells. The results indicate that combining *in vitro* immunization with phage display has advantages in generating human monoclonal antibodies.

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