

Efficacy of a recombinant single-chain fragment variable region, VasSF, as a new drug for vasculitis

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Background: Anti-neutrophil cytoplasmic autoantibodies (ANCA) associated vasculitis is a pauci-immune disease with the inflammation of the small blood vessels. The efficacies of antibody drugs for induction therapies of vasculitis vary among cases. Here, we developed a novel clone of a single chain Fv region (ScFv) with vasculitis-specific therapeutic potential.

Materials and methods: The clone, termed VasSF, was selected from our *Escherichia coli* expression library of recombinant human ScFv based on the therapeutic efficacy in an SCG/Kj mouse model of MPO-ANCA-associated vasculitis (MAAV), such as improvement of the urinary score and decreased crescent formation in glomeruli, granulomatous in lung, MPO-ANCA biomarkers, the anti-moesin antibody, and some cytokine levels.

Results: We identified vasculitis-associated apolipoprotein A-II (VAP2) as a target molecule of the clone and confirmed the independently-established VAP2 antibodies were also therapeutic in SCG/Kj mice. In MAAV, MPO-ANCA and cytokines stimulate neutrophils by facilitating heterodimer formation of VAP2 with apolipoprotein A-I in HDL.

Conclusion: VasSF would constitute a novel antibody drug for vasculitis by suppressing the heterodimer formation of the apolipoproteins.

Keywords: VasSF, ANCA antibody drug, apolipoprotein, HDL, myeloperoxidase, MPO, SCG/Kj, vasculitis

Introduction

Anti-neutrophil cytoplasmic autoantibodies (ANCA)-associated vasculitis, including anti-myeloperoxidase antibody (MPO-ANCA) and anti-proteinase 3 antibody (PR3-ANCA), might be caused by the injury of the blood vessels from the dysregulation of the activated neutrophils.¹ Vasculitis includes inflammation of various types of blood vessels. Patients with vasculitis show a wide variety of inflammatory signs in blood vessels of particular sizes. Guidelines for the treatment of vasculitis vary among Europe, the USA, and Japan in terms of the method to suppress inflammation. The European League against Rheumatism, the European Renal Association-European Dialysis and Transplant Association (ERA-EDTA), and the European Vasculitis Society, involving experts worldwide, reported a validation study for treating the induction of remission of ANCA-associated vasculitis and disease management. Immunosuppressive medications, such as cyclophosphamide and azathioprine, were recommended by almost all experts, and the combination with antibody drugs, eg, rituximab targeting CD20-positive cells, is also recommended for the treatment of granulomatosis with polyangiitis/microscopic polyangiitis.^{2,3} In Japan, the guidelines for treating severe vasculitis recommend steroids, immunosuppressive therapy, and antibody drugs, such as rituximab.³⁻⁵

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However, these therapies can cause severe infections in elderly patients with severe vasculitis. In addition, although antibody drugs likely work by binding to and neutralizing target antigens,⁶ it remains unclear how these drugs work in patients with severe vasculitis. Nevertheless, the efficacy of these antibodies suggests that vasculitis might be particularly treatable with therapeutic antibodies designed to target key molecules in the vasculitis pathway. Thus, antibody drugs for vasculitis treatment need to be developed based on a specific target molecule(s) as materials involved in the etiological mechanism of the disease. To improve therapeutic efficacy for MPO-ANCA-associated vasculitis (MAAV), we focused on the gamma globulin population in human blood because the administration of a large amount of intravenous gamma globulin (IVIg) is reportedly effective for treating patients with vasculitis.^{7–9} Although the effect of IVIg has been pointed out to be an immunomodulatory effect via Fc gamma (Fc γ) receptor, its therapeutic mechanism is not completely clarified.¹⁰ As Fc and sugar moieties of IgG can cause neutrophils activation via the Fc γ receptors,^{11,12} we have established a library of human, recombinant IgG, single-chain Fv regions (hScFv).¹³

Here, we developed a recombinant clone for a vasculitis-associated antibody drug by screening an hScFv library consisting of 204 clones based on the therapeutic activity in SCG/Kj mice¹³ that spontaneously develop vasculitis. The most effective clone was chosen for further study. The antibody's target molecule was identified by mass spectrometry (MS), and using the polyclonal antibodies its effectiveness was verified in SCG/Kj mice.¹⁴ Our findings will not only be useful for developing new antibody drugs for remission of MAAV, but also for understanding the etiology of vasculitis.

Materials and methods

Construction and screening of the recombinant hScFv library

Antibody fragments with a therapeutic benefit for vasculitis were selected from a human ScFv expression library containing 204 VH-CH1-hinge regions.¹³ The clones were classified according to the number of occurrences of identical VH region sequences in the library. Clones were grouped depending on the copy number of the same amino acid sequences of the VH regions, namely, four of the same clone (Group 1: Gr-1), three of the same clone (Group 2: Gr-2), and two of the same clone (Group 3: Gr-3). Unique clones were randomly selected (Group 4: Gr-4) (Figure S1).

Reconstruction and optimization of the insert

The most therapeutically effective clone, a Gr-1 Rq01 subclone, was sequenced, and the coding region was reconstructed for optimal expression in *Escherichia coli*. This reconstructed Rq01 (which we will refer to as URq01) was inserted into the pET-32a vector and transfected to BL21(DE3) competent cells. The sequence of the hScFv-expressing clone was confirmed via the vector construct (Figure S2A and B).

Purification of recombinant clone proteins

The recombinant URq01 (Gr-1) protein was induced with IPTG (0.1 M) overnight at 16°C and the cell pellets were harvested and frozen. The pellets were suspended in a 20 mL/L culture of 50 mM Tris (pH 8.0) containing 6 M guanidine thiocyanate, and sonicated at 20 kHz for 1 minute on ice with a VP-050 (TITEC, Gunma, Japan). The protein was purified from the extracts by affinity chromatography on a Ni-Sepharose 6 Fast Flow (GE Healthcare, Buckinghamshire, UK) column (2.5×8.0 cm) with 8 M urea, 0.3 M NaCl, and 50 mM Tris (pH 8.0). The proteins were eluted with 0.1 M EDTA, 8 M urea, 0.3 M NaCl, and 50 mM Tris (pH 8.0). The proteins were purified by gel filtration (high-performance liquid chromatography) on Shodex KW-802.5 (0.8×30 cm) and 803 (0.8×30 cm; SHOWA DENKO K.K., Tokyo, Japan) columns and gel-filtration column chromatography with 8 M urea in PBS. The proteins were further purified by a (TSKgel®) DEAE-5PW column (0.75×7.5 cm; TOSOH, Tokyo, Japan), and by cobalt-coordinate Sepharose 6 Fast Flow. The protein was concentrated and applied to gel filtration to exchange the 6 M guanidine in the PBS (Figure S2C–E). The hScFv was renatured by removing the guanidine by dialysis with PBS containing 0.8 M arginine and subsequent dialysis with PBS only.

Evaluation of therapeutic effects in a mouse model

All animal experiments followed the guidelines from the National Institutes of Biomedical Innovation, Health, and Nutrition, Osaka, Japan (authorization number: DS21-8). Therapeutic effects were evaluated using SCG/Kj mice as the MAAV mouse model.¹⁴ In the treatment group, 10-week-old mice were treated daily for 5 days with intraperitoneal injections of 0.4 mg/kg of samples dissolved in PBS-containing stabilizers (1.5% D-mannitol and 0.45% glycine). Mice in the control group were administered solvent only. The numbers of mice in experimental groups were 6, 4, 3, 3, 3, 4, and 4 in

C57BL/6, solvent, G1LQq, G1hEq, G2Lt, VasSF, hIgG, and vasculitis-associated apolipoprotein A-II (VAP2), respectively. The animals were euthanized by CO₂ 3 weeks later.

Effect of treatment on the SCG/Kj mice

Urinary scores ranging from 0 to 7 were sums of the urinary protein and occult blood scores measured by the Uropaper III Eiken E-UR42 (Eiken Chemical Co. Ltd., Tokyo, Japan). The urinary protein scores were as follow: 0, if urinary protein concentration measured by a protein error of pH indicators¹⁵ <30 mg/dL; 1, if 30–100 mg/dL; 2, if 100–300 mg/dL; 3, if 300–1,000 mg/dL; and 4, if >1,000 mg/dL. Note that a considerable amount of urinary protein (20–30 mg/dL) is usually detected even in normal mice because of the presence of major urinary proteins.¹⁶ The occult blood levels were classified from 0 (not detected) to 3 (high concentration). The crescent formation rate (% crescents) was determined with 80–100 glomeruli on a hematoxylin and eosin-stained slide of kidney tissues. Blood counts of white blood cells, lymphocytes, monocytes, and granulocytes were measured with a VetScan HMII auto analyzer (ABAXIS, Union City, CA, USA). The serum levels of biomarkers, MPO-ANCA, and anti-moesin antibodies^{17,18} were measured by enzyme-linked immunosorbent assay. The values of MPO-ANCA titer was shown as the equivalent of the standard mouse MPO-ANCA serum IgG. Multiplex immunoassays (Bio-Plex; Bio-Rad, Hercules, CA, USA) were used to measure serum levels of 23 inflammatory cytokines/chemokines,¹⁷ that is, interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor, granulocyte macrophage-colony stimulating factor, interferon (IFN)- γ , keratinocyte-derived chemokine, monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP) – 1 α , MIP-1 β , regulated on activation, normal T cell expressed and secreted, and tumor necrosis factor- α (TNF- α).

Determination of the target molecules

We selected the most effective clone, named VasSF (vasculitis-associated ScFv), to identify its target molecule(s) by affinity purification followed by MS. One milligram of VasSF was applied to 25 mg of Dynabeads Tosylactivated (Thermo Fisher Scientific, Waltham, MA, USA) in 0.1 M sodium borate buffer, pH 9.5. The VasSF-bound beads were washed five times with PBS containing 0.05% Tween 20. Three milliliters of plasma from healthy volunteers was diluted 10-fold with PBS and incubated with 25 mg of

the beads with rotation for 1 hour at room temperature. The bound proteins were eluted with 0.1 M glycine-HCl, pH 2.7, and the eluate was neutralized by adding 1:10 vol of 1 M TrisHCl, pH 9.0. Then, the eluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel region was visualized by silver staining and was excised. The bound peptide fragments were released from the excised piece of gel by digestion with pro-tease. The peptide fragments were subjected to MS. Tandem mass spectroscopy (MS/MS) spectra were obtained by selecting ions from specific peptides in the mass spectrometer. Five milligrams of VAP2-conjugated beads were prepared as described above. The beads were mixed with 3 mL of the VasSF solution and washed seven times. The bound proteins were eluted and detected by Western blotting with the anti-human IgGF (ab') 2 antibody (609-4118, Rockland Immunochemicals Inc., Limerick, PA, USA).

Statistical analyses

The urinary scores were statistically analyzed with the Kruskal–Wallis analysis of variance by ranks, followed by Dunn's method using SigmaPlot version 14 (Systat Software Inc., San Jose, CA, USA). The other observed values were statistically analyzed with analysis of variance, followed by Tukey's test if normality and equal variance were confirmed by the Shapiro–Wilk and Brown–Forsythe tests, respectively. Otherwise, statistical analyses were performed using the Kruskal–Wallis one-way analysis of variance by ranks, followed by Dunn's test. A *P*-value <0.05 was considered significant. The observed values that showed the normal distribution were described as the mean $\pm$ SD, otherwise described as the median (min – max).

Results

Selection of a clone from the hScFv 204-clone library

The selected clone groups were divided into four groups: Gr-1, Gr-2, Gr-3, and Gr-4 (Figure S1). The sequence encoding VasSF was subcloned to optimize expression in *E. coli*. One Gr-1 clone (subclones Rq01, G1LQq, G1Qq, and G1hEq) was selected based on the therapeutic efficacy of the purified protein injected into SCG/Kj mice. Efficacy was based on 1) urinary score (Figure 1A), 2) crescent formation in glomeruli (Figure 1B), 3) spleen weight/body weight (Figure 1C), 4) histological status of the kidney and spleen (not shown), and 5) serum levels of biomarkers such as MPO-ANCA (Figure 1D), TNF- α (Figure 1E), and anti-moesin antibody (Figure 1F).

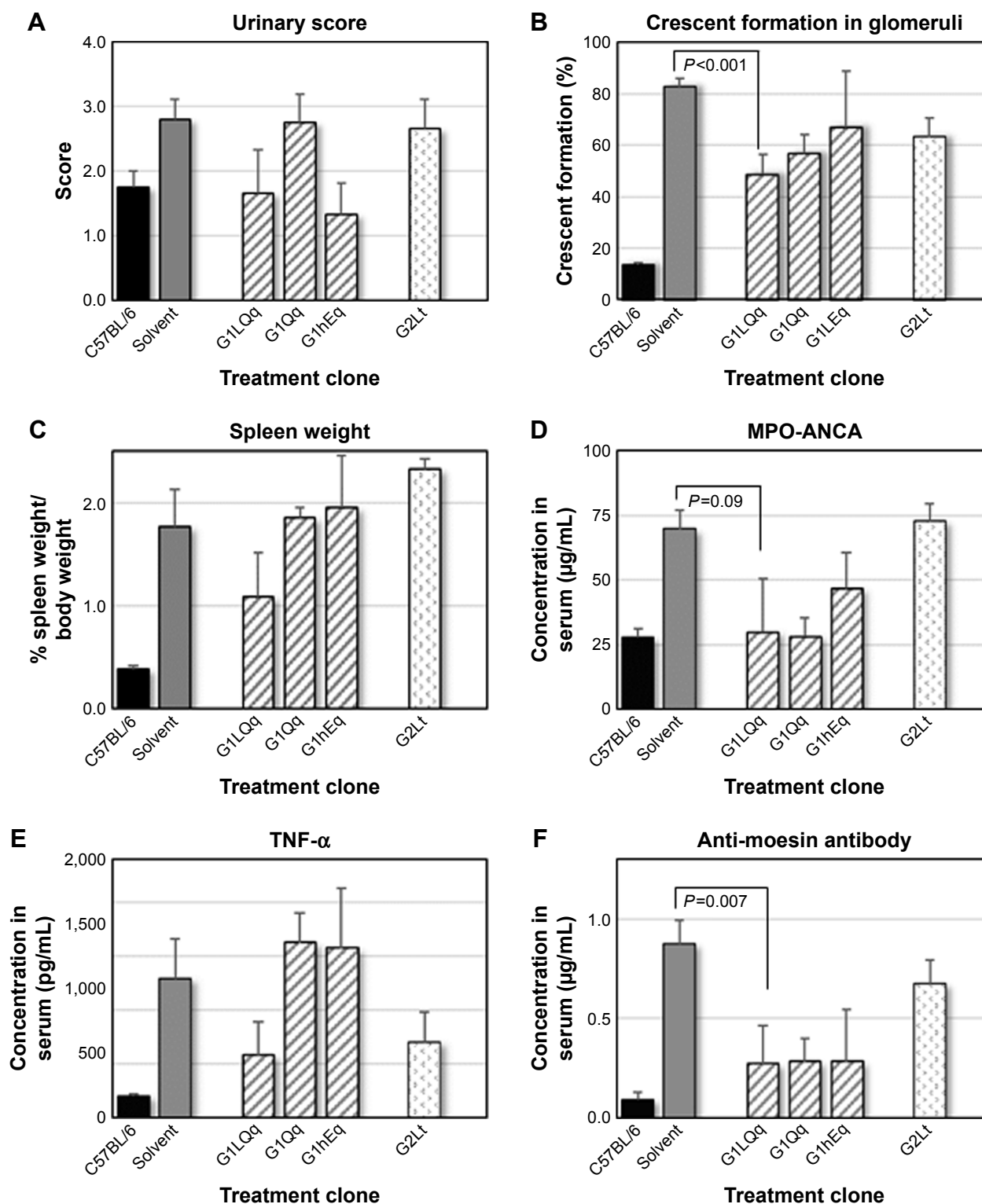


Figure 1 Selection of the therapeutic hScFv clone from the 204-clone library.

Note: Evaluation criteria were the urinary score (A), crescent formation (B), spleen weight/body weight (C), serum biomarker levels of MPO-ANCA (D), TNF-α (E), and an antibody to moesin (anti-moesin, F).

Abbreviations: hScFv, human single-chain Fv regions; MPO-ANCA, myeloperoxidase anti-neutrophil cytoplasmic autoantibodies; TNF-α, tumor necrosis factor-α.

VasSF injections had a therapeutic effect on kidney and immune functions

Significantly more glomerular crescent formation was observed in SCG/Kj mice administered control samples

(ie, “solvent”) than that of the healthy control C57BL/6 mice, but VasSF treatment reversed a variety of these disease manifestations in mice compared with those in mice in the “solvent” control group: the crescent formation recovery value

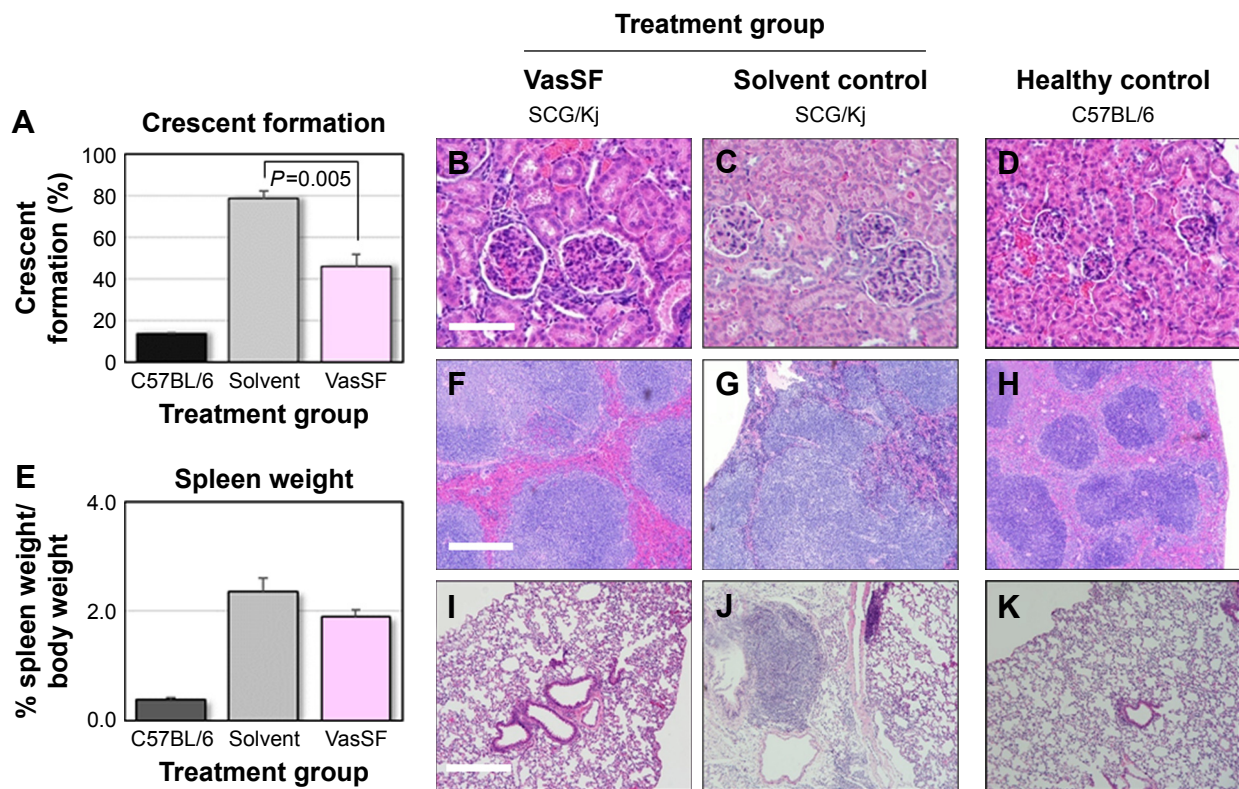


Figure 2 Therapeutic effects of VasSF on kidney, spleen, and lung.

Notes: Percentage crescent formation in glomeruli. (A) Microscopic analysis of a kidney in an SCG/Kj model mouse after administration of the purified recombinant VasSF therapeutic (B), the solvent control (C), or a representative healthy C57BL/6 mouse. (D) Spleen weight ratio. (E) Microscopic analysis of spleen in an SCG/Kj model mouse after administration of the purified recombinant VasSF therapeutic (F), the solvent control (G), or a representative healthy C57BL/6 mouse (H). Microscopic analysis of a lung in an SCG/Kj model mouse after administration of the purified recombinant VasSF therapeutic (I), the solvent control (J), or a representative healthy C57BL/6 mouse (K). Bar: 100 μ m.

Abbreviation: VasSF, vasculitis-associated ScFv.

was $46.1\% \pm 11.3\%$ from $78.5\% \pm 10.2\%$ (Tukey, $P=0.005$) in “solvent” (Figure 2A). The microscopic observations of the VasSF treatment showed recovery of glomeruli (Figure 2B) compared with that for the solvent control (Figure 2C) as well as healthy control (Figure 2D). In addition, spleen weight/body weight was 1.85% ($1.64\%–2.24\%$) from 2.36% ($1.64\%–3.13\%$) (Dunn, $P=1.00$) in solvent (Figure 2E), and microscopic separations of spleen white and red pulp clear separated the VasSF treatment (Figure 2F) from the solvent (Figure 2G) as shown in the healthy control (Figure 2H). Moreover, microscopic observations of lungs showed that VasSF treatment recovered normal lungs (Figure 2I) from those in the “solvent” control (Figure 2J), as shown in the C57BL/6 healthy control (Figure 2K).

Furthermore, animals given the VasSF therapy showed improved serum biomarker profiles in comparison with those in the control group: MPO-ANCA (37.7 ± 11.2 vs 61.3 ± 19.2 μ g/mL; Tukey, $P=0.168$; Figure 3A), anti-moesin antibody (0.13 ± 0.25 vs 0.75 ± 0.33 μ g/mL; Tukey, $P=0.011$; Figure 3B), and TNF- α (491 [168–887] vs $1,360$ [489–2,435] pg/mL; Dunn, $P=0.652$; Figure 3C).

Serum profiles of inflammatory cytokines/chemokines also improved, ie, IL-1 β : 386 (170–968) vs $3,829$ (837–6,808) pg/mL, Dunn, $P=0.023$, Figure 3D; IL-5: 30.3 (14.7–82.7) vs 250 (60.0–373) pg/mL, Dunn, $P=0.028$, Figure 3E; IL-17: 42.3 (20.8–52.6) vs 170 (96.7–568) pg/mL, Dunn, $P=0.007$, Figure 3F; IFN- γ : 27.7 (15.8–84.0) vs $1,007$ (283–1,977) pg/mL, Dunn, $P=0.017$, Figure 3G; and MIP-1 β : 73.4 (14.2–115) vs 481 (218–695) pg/mL, Dunn, $P=0.008$, Figure 3H.

Vasculitis-associated apolipoprotein A-II for the VasSF antigen/target molecule

Affinity pull down with the Tosylactivated Dynabeads coated with VasSF isolated a clear band at ~ 10 kDa (red arrowhead in Figure 4A). The cutout piece of the gel was estimated to contain eleven candidate proteins including VAP2 registered in the database. In contrast, binding VasSF to VAP2 was confirmed by binding with VAP2-conjugated beads (Figure 4B). We also investigated the 25 kDa band, which was apolipoprotein A-1 (AP1) according to the MS/MS analysis (Figure 4A, open arrowhead), showing VAP2 binding AP1 in serum.

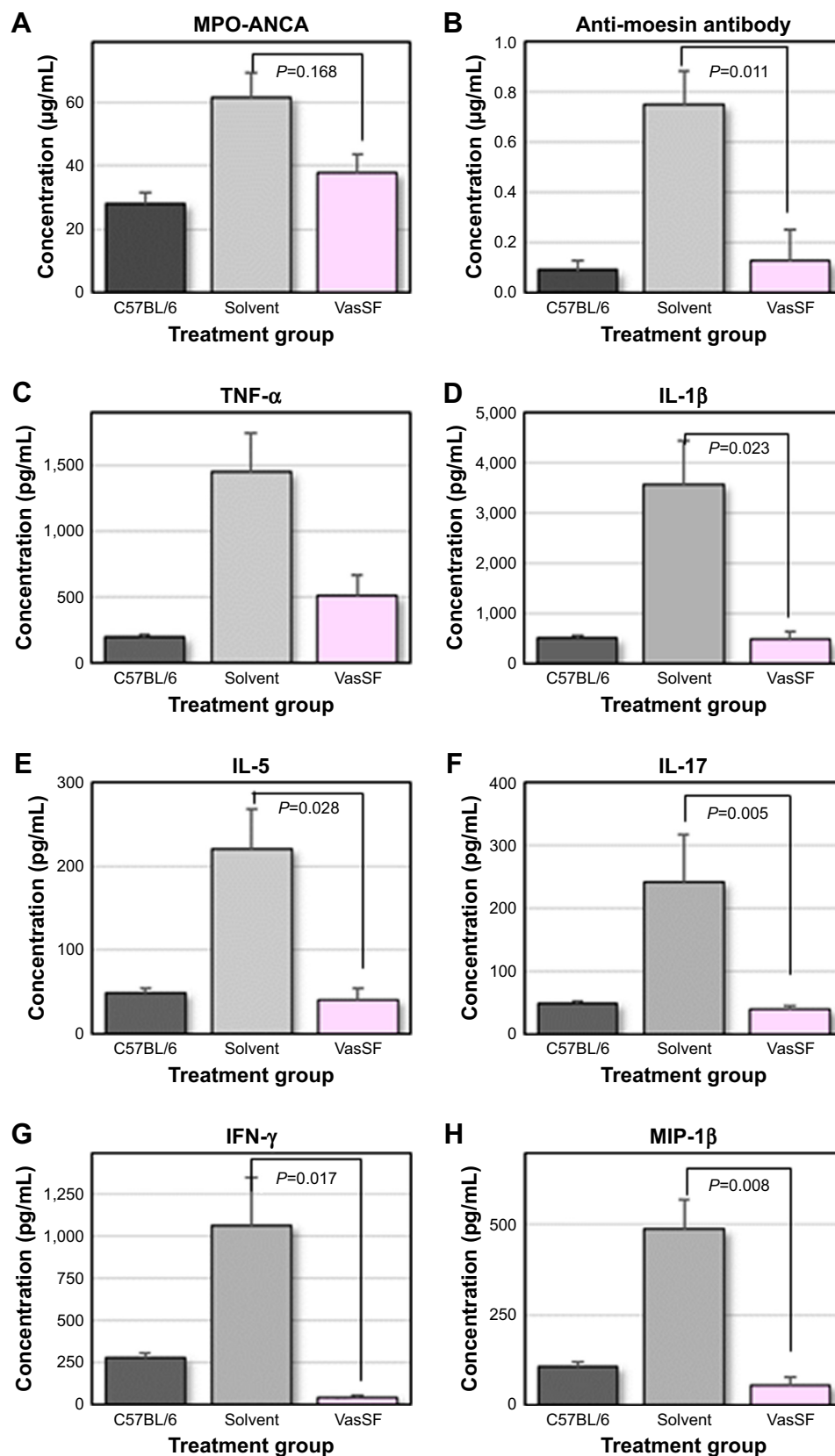


Figure 3 Therapeutic effects of VasSF on two biomarkers (MPO-ANCA and anti-moesin antibody), and cytokines/chemokines in serum.

Note: Biomarkers and cytokines/chemokines were measured in serum from healthy control (C57BL/6) and SCG/Kj mice treated with solvent only (solvent; control), and SCG/Kj mice treated with the purified recombinant VasSF therapeutic (VasSF): MPO-ANCA (A), anti-moesin antibody (B), TNF-α (C), IL-1β (D), IL-5 (E), IL-17 (F), IFN-γ (G), and MIP-1β (H).

Abbreviations: IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; MPO-ANCA, myeloperoxidase anti-neutrophil cytoplasmic autoantibodies; TNF-α, tumor necrosis factor-α; VasSF, vasculitis-associated ScFv.

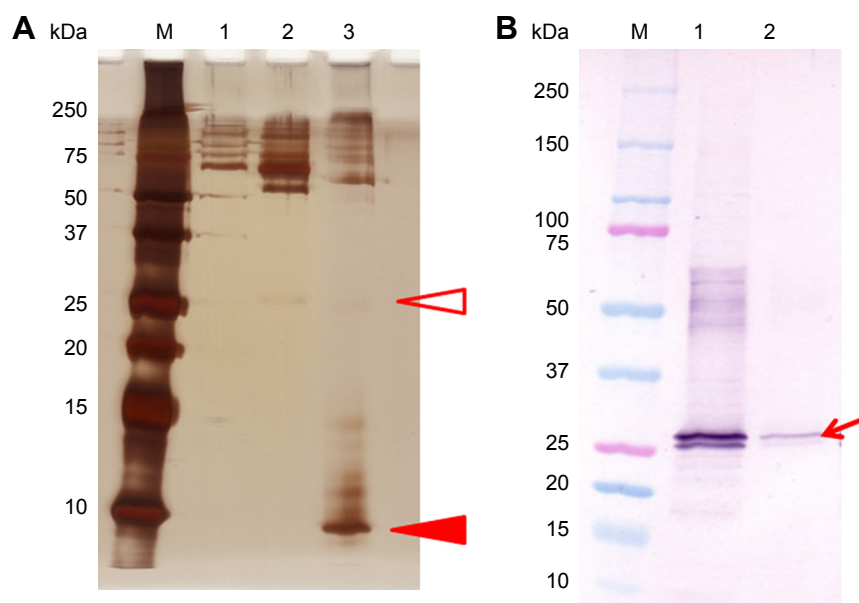


Figure 4 Identification of VasSF target molecules.

Notes: (A) SDS-PAGE with silver stain; lane M, molecular weight markers; lane 1, plasma 50 kD filter flow-through fraction; lane 2, unbound fraction of plasma exposed to VasSF beads; lane 3, eluate fraction of plasma bound to the VasSF beads. Red arrowheads, the portion of the gel cut out for MS/MS analysis. (B) Western blot detection (red arrow) with anti-Fab antibody of VasSF binding to VAP2-conjugated beads; lane M, molecular weight markers; lane 1, VasSF solution before pull down by VAP2-conjugated beads; lane 2, fraction eluted from VAP2-conjugated beads.

Abbreviations: VAP2, vasculitis-associated apolipoprotein A-II; VasSF, vasculitis-associated ScFv.

We wanted to determine whether VAP2 is a VasSF antigen that binds VAP2 in human plasma in vitro. Therefore, anti-VAP2 polyclonal antibodies (aVAP2) were injected into SCG/Kj mice to alleviate their vasculitis symptoms, such as glomerular crescent formation (from $78.5\% \pm 5.1\%$ to $56.3\% \pm 6.3\%$; $P=0.078$; Figure 5A). The recovery of the glomerular crescent formation with the aVAP2 was also confirmed in histological observations of the aVAP2 treatment (Figure 5B) compared to the solvent (Figure 5C) and the hIgG (Figure 5D). Moreover, biomarkers and cytokines recovered by the VasSF treatment were also analyzed with the aVAP2. The following biomarkers were measured in aVAP-treated and solvent-control groups: MPO-ANCA (38.9 ± 16.6 vs 61.3 ± 19.2 $\mu\text{g/mL}$; Tukey, $P=0.204$; Figure 5E), anti-moesin antibody (0.11 ± 0.22 vs 0.75 ± 0.32 $\mu\text{g/mL}$; Tukey, $P=0.009$; Figure 5F), and TNF- α (374 [321–708] vs 1,360 [489–2,435] pg/mL ; Dunn, $P=1.00$; Figure 5G). Serum levels of cytokines were measured: IL-1 β 634 (556–728) vs 3,829 (837–6,808) pg/mL ; Dunn, $P=0.168$; Figure 5H, IL-5 57.0 (28.5–69.9) vs 250 (60.0–373) pg/mL ; Dunn $P=0.225$; Figure 5I, IL-17 74.1 (40.1–78.2) vs 170 (96.7–568) pg/mL ; Dunn, $P=0.342$; Figure 5J, IFN- γ 38.4 (14.0–49.0) vs 1,007 (283–1,977) pg/mL ; Dunn, $P=0.009$; Figure 5K and MIP-1 β 76.5 (43.3–87.6) vs 481 (218–695) pg/mL ; Dunn, $P=0.031$; Figure 5L. The number of peripheral blood lymphocytes and granulocytes did not change in the aVAP2-administered mice: WBCs: $4.42 (1.36–7.57) \times 10^9$ cells/L vs

$3.14 (1.48–15.7) \times 10^9$ cells/L (Figure S3A). The number of granulocytes decreased: $0.15 (0.05–1.04) \times 10^9$ cells/L vs $0.22 (0.04–0.69) \times 10^9$ cells/L (Figure S3B).

These results indicate that VasSF targeted VAP2, and that the effective dose of VasSF was 1:4,000 of IVIg. Furthermore, the high amino acid sequence homology between VasSF (clone URq01) and the anti-VAP2 mouse monoclonal antibody registered in the public database (Figure S4) also indicated that the VasSF target antigen was VAP2. The idiotype (antigen binding site) in the two VH domains showed homologous amino acid alignment in 18/48 and 14/29 in the structure of the heavy chain variable region.

Discussion

In this study, we isolated a novel therapeutic clone from a 204-clone hScFv library.¹³ Our strategy for selection was quite unique from that used to develop other antibody drugs, which recognize etiological and pathogenic molecules, such as cytokines/chemokines and their receptors. Two popular new antibody drugs, infliximab, the efficacy of which has been reported,¹⁹ and rituximab,^{3–5,20,21} have been developed as humanized IgG antibodies containing the entire antibody structure. As Fc and sugar moieties could cause neutrophils activation via the Fc γ receptor,^{11,12} we devised a strategy to eliminate these moieties, and selected a single clone from an ScFv fragment library of polyclonal IgGs.¹³ Our antibody fragment not only recognized a protein target, but was also effective for MAAV.¹³

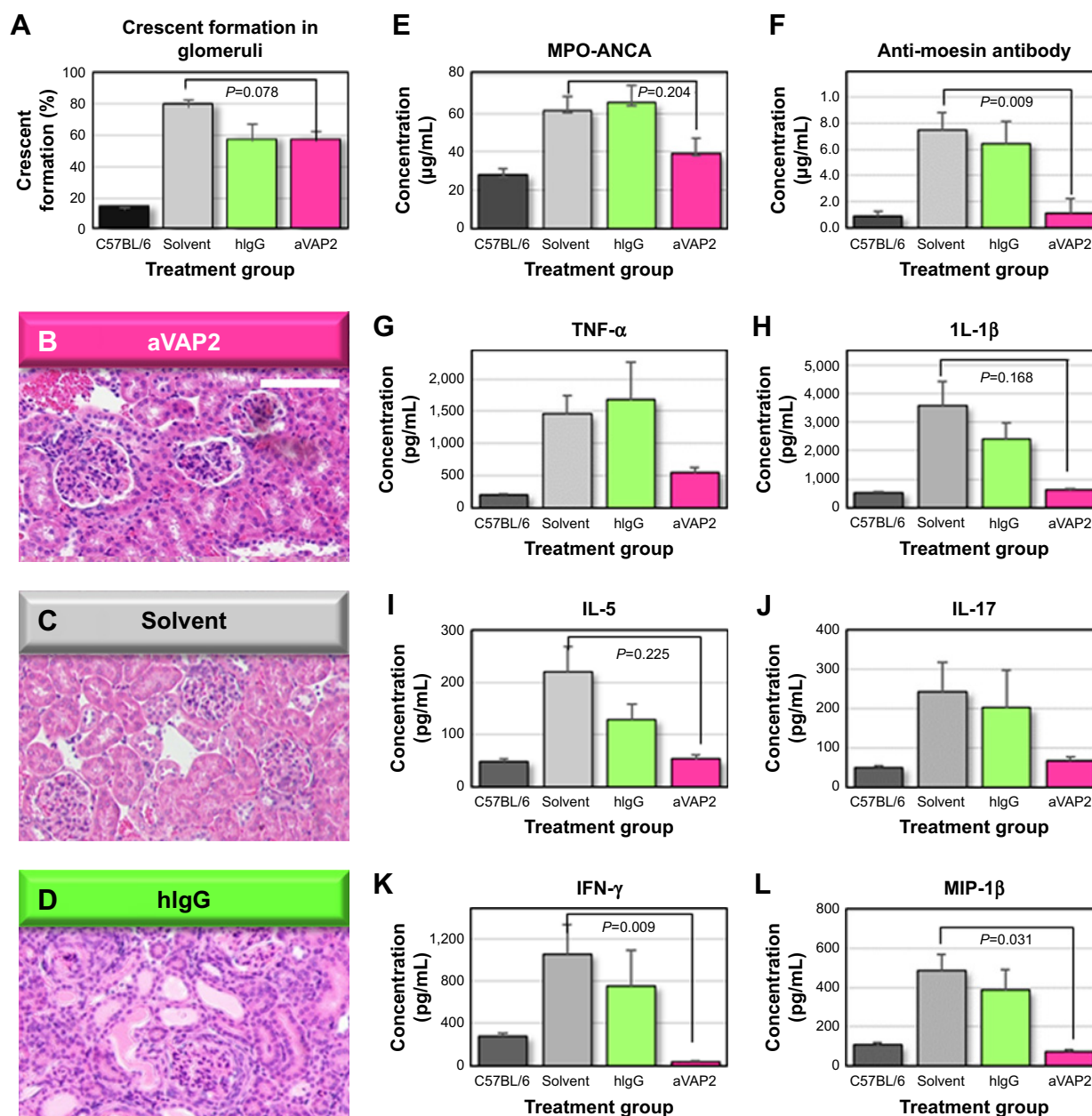


Figure 5 Therapeutic effects of aVAP2 in mouse models of MAAV.

Notes: Administration of the aVAP2 to SCG/Kj model mice was therapeutic for crescent formation, serum biomarkers of MPO-ANCA, anti-moesin antibodies, and cytokines/chemokines: Glomerular crescent formation rates (%) in kidneys (A). Microscopic observations of kidneys treated with aVAP2 (B), solvent as a solvent control (C), and hlgG as an immunoglobulin control (D). Serum levels of MPO-ANCA (E), anti-moesin antibody (F), TNF- α (G), IL-1 β (H), IL-5 (I), IL-17 (J), IFN- γ (K), and MIP-1 β (L). Bar: 100 μm .

Abbreviations: aVAP2, anti-VAP2 polyclonal antibodies; IFN, interferon; IL, interleukin; MAAV, MPO-ANCA-associated vasculitis; MIP, macrophage inflammatory protein; MPO-ANCA, myeloperoxidase anti-neutrophil cytoplasmic autoantibodies; TNF- α , tumor necrosis factor- α .

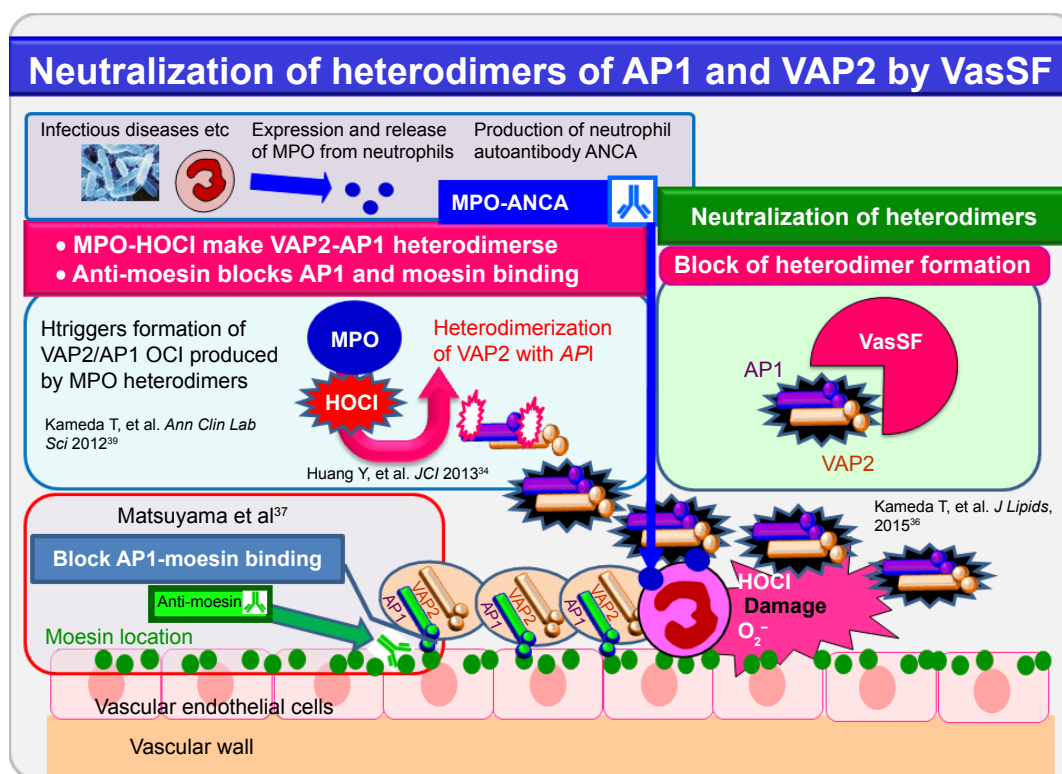
Structural modification of therapeutic antibodies from their complete forms of immunoglobulins to target-binding antibody fragments may be a major step in the development of antibody drugs. Studies have increasingly focused on the ScFv region only, which binds antigen(s), especially for cancer treatment and immunotherapy.^{22,23} For example, the CTLA-4 antibody ScFv is projected to be an effective new antibody-type drug.²⁴

We not only isolated a therapeutic antibody fragment clone with therapeutic effects on a mouse MAAV vasculitis model, but also identified its VAP2 target antigen molecule. Furthermore, the VasSF (URq01 clone) amino acid sequence showed high homology with the H-chain VH region of a mouse monoclonal antibody against VAP2 (Accession No NP_001634). The sequence was not found in any registered human gene sequences, including that of infliximab²⁵ or

oxidative action of HOCl produced by MPO released from neutrophils.^{32–35} The HOCl facilitates formation of the VAP2-AP1 heterodimer in HDL and decreases its anti-inflammatory activity. These molecular interactions suggest that VasSF might be therapeutically effective not only in vasculitis, but also in cardiovascular diseases.

Conclusion

Notably, however, the VasSF target antigen, VAP2, associates with AP1 to form high-density lipoprotein (HDL), which has anti-inflammatory action.³² It is known that AP1 and AP2 in HDL undergo a structural change due to the



Abbreviations: VasSF, vasculitis-associated ScFv; VAP2, vasculitis associated apolipoprotein A2; APl, apolipoprotein A1.

to trigger the formation of heterodimers due to an imbalance between AP1 and AP2 in HDL.³⁸ This may result in damage to endothelial cells. Adding the anti-VAP2 antibody fragments, VasSF, might recover MAAV by suppressing this heterodimer formation in peripheral blood.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

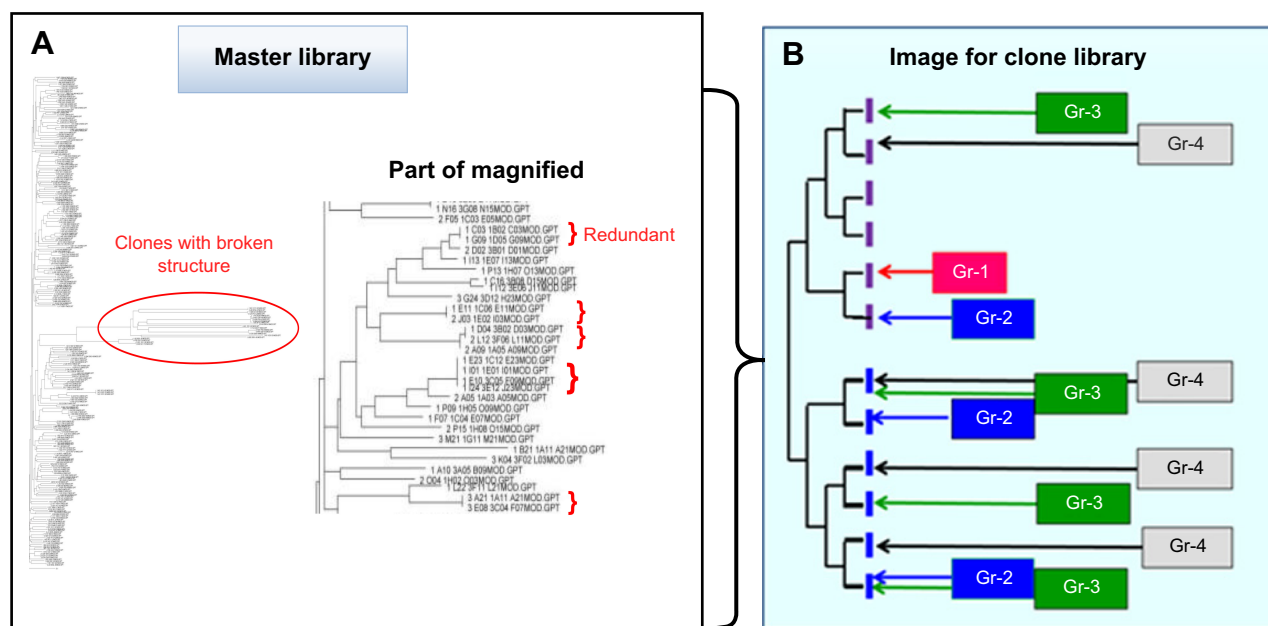


Figure S1 Plasmid preparation for hScFv from the 204-clone hScFv library.

Notes: Part of the phylogenetic tree of hScF clones in our library (A). Schematic display of clones selected from our hScFv library (B).

Abbreviation: hScFv, human single chain Fv region.

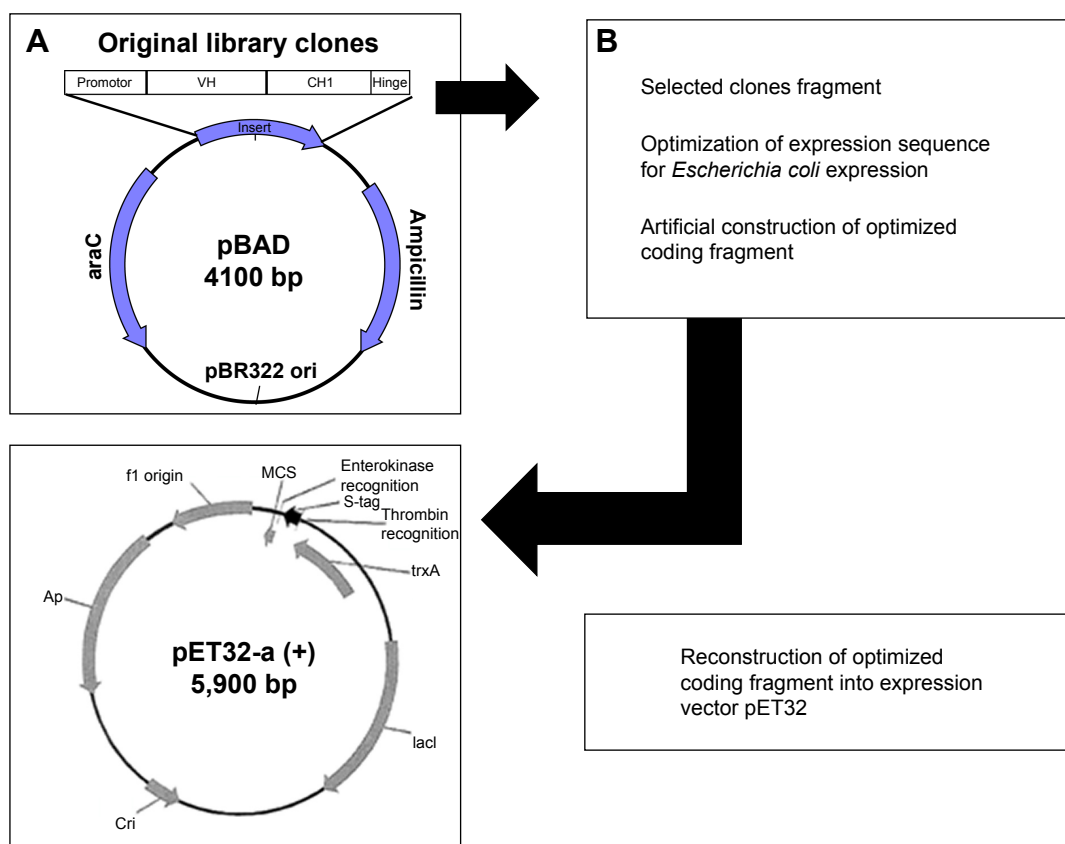


Figure S2 (Continued)

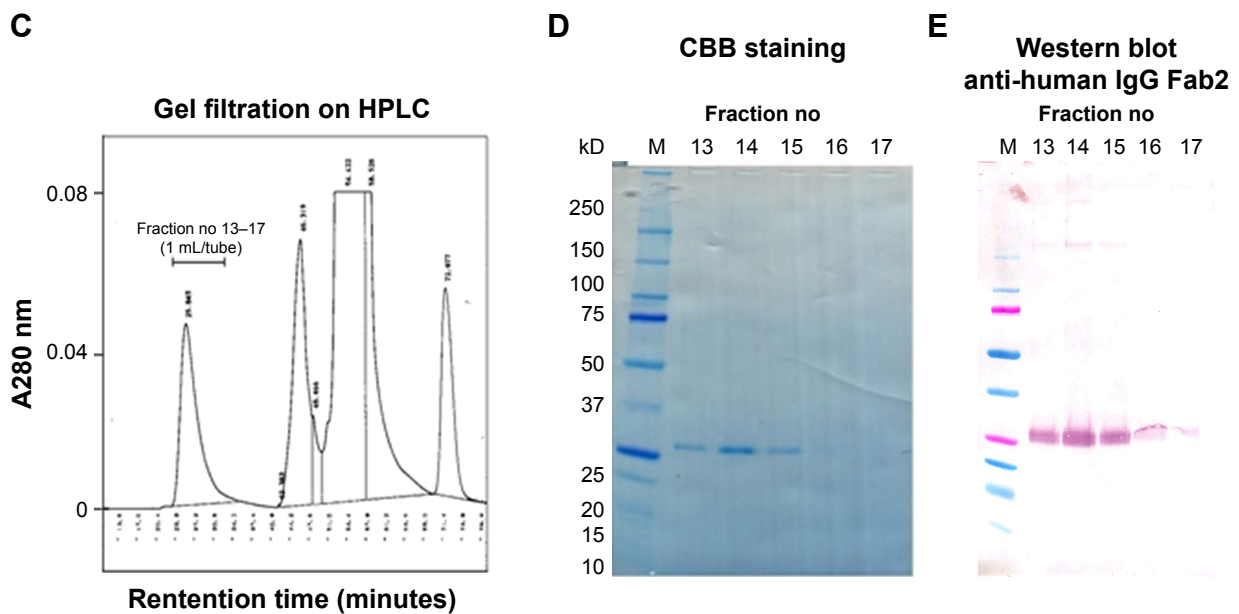


Figure S2 Plasmid preparation for hScFv and the purification procedures.

Notes: The original hScFv library was constructed in the pBAD expression vector (A). The selected clones were optimized for *Escherichia coli* expression and inserted in pET32-a (B). Gel filtration separation profile of hScFv (C). CBB stain of isolated hScFv on SDS-PAGE (D). Western blot analysis of hScFv by anti-human Fab2 antibody (E).
Abbreviation: hScFv, human single-chain Fv regions.

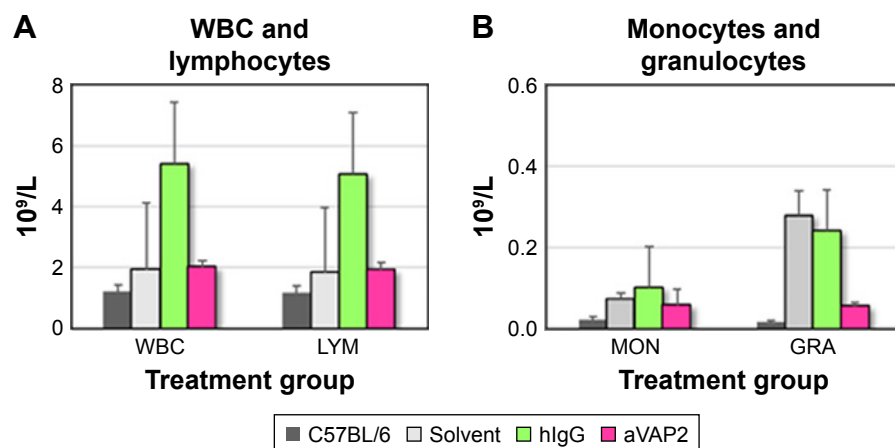


Figure S3 Blood cell counts.

Notes: Counts of WBC, LYM, MON, and GRA in peripheral blood are compared among healthy control C57BL/6, solvent, hlgG-treated, and aVAP2-treated mice in panels (A and B).

Abbreviations: aVAP2, anti-VAP2 polyclonal antibodies; GRA, granulocytes; LYM, lymphocytes; MON, monocytes; WBC, white blood cells.

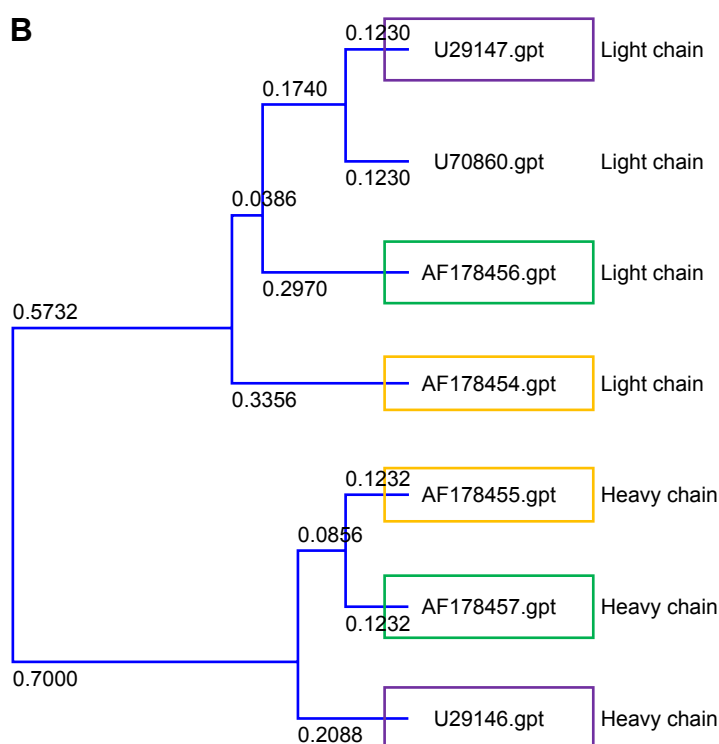
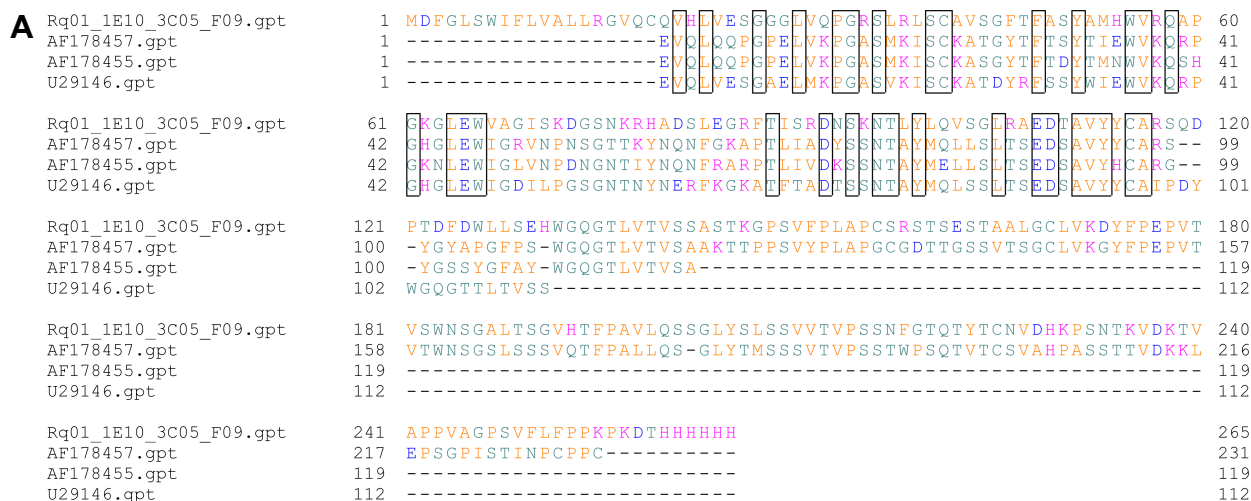


Figure S4 Alignment of amino acid sequence of the variable region among anti-VAP2.

Notes: Alignment of amino acid sequence of the variable regions among anti-apolipoprotein monoclonal antibodies and URq01 (**A**). Phylogenetic analysis of amino acid sequence of the registered variable region of anti-VAP2 monoclonal antibodies (**B**).

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