A novel and effective approach to generate germline-like monoclonal antibodies by integration of phage and mammalian cell display platforms

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Phage display technology allows for rapid selection of antibodies from the large repertoire of human antibody fragments displayed on phages. However, antibody fragments should be converted to IgG for biological characterizations and affinity of antibodies obtained from phage display library is frequently not sufficient for efficient use in clinical settings. Here, we describe a new approach that combines phage and mammalian cell display, enabling simultaneous affinity screening of full-length IgG antibodies. Using this strategy, we successfully obtained a novel germline-like anti-TIM-3 monoclonal antibody named m101, which was revealed to be a potent anti-TIM-3 therapeutic monoclonal antibody via in vitro and in vivo experiments, indicating its effectiveness and power. Thus, this platform can help develop new monoclonal antibody therapeutics with high affinity and low immunogenicity.

Keywords: phage display; mammalian cell display; germline-like; monoclonal antibody; TIM-3

INTRODUCTION
Over the past decades, monoclonal antibodies (mAbs) have become the most important class of therapeutic biologicals on drug market [1, 2]. The development of mAbs remains a key issue in meeting the world’s profound need for biological drugs [3]. Among therapeutic antibodies, antibody affinity plays an important role in biological efficacy [4], and higher antibody affinity typically allows for lower dosage. Generating high-affinity antibodies against important drug targets for clinical use remains a challenging task.

Development of therapeutic antibodies has been accelerated by in vitro antibody selection technologies, which permits rapid generation of millions of clones [5–8]. Phage display is the first and the most widely used platform for the discovery of fully human antibodies [9, 10]. It allows for affinity enrichment selections from an extremely large collection of antibodies with sequences of up to 1011 in size [11–14]. One inherent limitation of phage display, however, is that panning a non-immune phage display antibody library sometimes results in the selection of antibodies with $K_D$ ranges >100 nM [15, 16], so it is frequently not sufficient for effective clinical use [16, 17]. Furthermore, antibodies expressed on phages do not undergo normal mammalian posttranslational modifications. This drawback renders a step of conversion to whole IgG molecules and the expression of the candidate clones in mammalian cells necessary for further characterization of their biological activities. Most importantly, the panning process of phage display selection is a black box process, which cannot guarantee the quality of the output clones during the process [18–20]. A possibility to address one of these issues is to use mammalian cell display, which possesses intrinsic abilities to fold and glycosylate full-length IgG [21]. It enables the display of whole IgG and selection of positive clones with high affinity and other specific biological functions by fluorescence-activated cell sorting (FACS) [7, 8, 22–24], allowing for highly controlled and real-time selection of IgG. These properties render mammalian cell display extremely attractive and potent for antibody therapeutics development. However, one major limitation of this method is the reduced library diversity it allows compared to phage display [8]. The lower transformation efficiency of mammalian cells has been considered a significant barrier to the construction of highly diverse libraries.

As described above, both phage and mammalian cell display have inherent limitations that preclude the successful selection of antibodies with desired affinities and low immunogenicity. To efficiently screen for fully functional IgG antibodies against specific targets, we sought to integrate the phage and mammalian cell platforms by combining a pre-enriched antibody output and a FACS step for selection. In general, conventional mammalian cell libraries are generated by directly inserting DNA of antibodies into a mammalian cell vector. On the contrary, the workflow of our new platform starts with a screening and preselection step in a phage library. First, two rounds of phage library panning are performed, and the pre-enriched polyclonal antibody plasmid DNA from the last selection output is purified and cloned into...
To validate this new technology, we set out to generate antibodies with high binding affinity for T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), which is a promising candidate for cancer immunotherapy [25, 26] that was first reported in 2002 [27]. In this study, by using our newly developed platform, we obtained a novel anti-TIM-3 germline-like mAb named m101, which was found to possess high binding affinity, specificity, and potency in in vitro and in vivo experiments. We show that this novel approach, which integrates phage and mammalian cell display platforms, can overcome the limitations of said platforms and allow effective and reliable selection of full-length IgG antibodies with germline-like sequences and high binding affinity.

MATERIALS AND METHODS

Mice
Six- to eight-week-old female TIM-3 humanized C57BL/6N mice were obtained from the Nanjing Galaxy Biopharma Company, China. Six- to eight-week-old male NSG mice were obtained from Shanghai Model Organisms Center, China. The mice were housed under specific pathogen-free conditions in the Animal Care Facility of Fudan University. All the animal studies were carried out in accordance with institutional guidelines.

Cell line
TIM-3-overexpressing FCHO cells were generated in our laboratory. Human embryonic kidney Expi 293F cells were purchased from Thermo Fisher. Murine colon cancer cell line MC38, Chinese hamster ovary cell line, and human lymphoma Raji cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences.

Cell culture
Human lymphoma Raji cells were maintained in RPMI-1640 culture medium (Hyclone) with 10% fetal calf serum (Gibco), 100-units/mL penicillin (Gibco), and 100-μg/mL streptomycin (Gibco), in a 37°C incubator with 5% CO2. Murine colon cancer cell line MC38 was maintained in DMEM culture medium (Hyclone) with 10% fetal calf serum (Gibco), 100-units/mL penicillin (Gibco), and 100-μg/mL streptomycin (Gibco), in a 37°C incubator with 5% CO2. TIM-3-overexpressing FCHO cells were maintained in F12 culture medium (Hyclone) with 10% fetal calf serum (Gibco), 100-units/mL penicillin (Gibco), and 100-μg/mL streptomycin (Gibco), in a 37°C incubator with 5% CO2. The cells were expanded in T-175 flasks, harvested by trypsinization on the day of inoculation. The cell viability was tested with Trypan Blue staining and was >95% during the incubation.

Protein and antibody expression
Overlapping PCR was used to construct a cDNA encoding a fusion protein with TIM-3 extracellular domain and human Ig Fc fragment. The overlapping PCR product was cloned into a PT expression vector. Plasmids for antibody expression were from E. coli and were used for transfection and protein expression. Expi 293F cells were transfected, and the proteins secreted in the medium were purified by Protein A affinity chromatography (GE Healthcare). The homogeneity and purity of the protein preparations were verified by SDS-PAGE. Protein concentration was measured by reading 280-nm absorbance.

Both the H and L chain genes of anti-TIM-3 antibodies were cloned into PT expression vectors. The H and the L chain vectors were co-transfected into 293F cells using the Expi293 expression system (Thermo Fisher Scientific) following the manufacturer’s instructions. Then, IgGs were purified by Protein A affinity chromatography (GE Healthcare). Proteins were dialyzed against phosphate-buffered saline (PBS). Protein concentration was here measured with a microplate reader (Biotek).

Screening of anti-TIM-3-Fc mAb from phage display library
First, we used a large phage display naïve human Fab library constructed by using mixed PBMC cDNAs from 40 healthy volunteers to achieve a titer of $1.5 \times 10^{11}$ [28]. TIM-3-Fc-biotin and streptavidin (SA) beads were used for two rounds of magnetic selection. Phages from the library were pre-blocked in 3% milk powder (w/v) in PBS (MPBS) and incubated with the TIM-3-Fc-biotin in 1% MPBS for 30 min. Next, SA magnetic beads were added and incubated for 1.5 h. The tubes were washed with PBS containing 0.05% Tween 20 (PBST). TIM-3-Fc binding phages were used to infect mid-log phase TG1 E. coli at 37°C for 1 h. Then, TG1 bacteria were grown in 2YT medium containing 100-mg/mL ampicillin and 2% (w/v) glucose at 37°C. After 2 h, the cells were infected with M13KO7 helper phages (Invitrogen) for 45 min at room temperature. The infected cells were harvested and resuspended into 2YT medium supplemented with 100-mg/mL ampicillin and 100-mg/mL kanamycin, then incubated overnight at 30°C. The phages were precipitated from the culture supernatant with PEG8000-NaCl and resuspended in sterile PBS for subsequent panning. The enrichment for antigen-specific phages after each round of panning was assessed by polyclonal phage enzyme-linked immunosorbent assay (ELISA).

ELISA
The binding capacity of anti-TIM-3 IgGs was measured by ELISA. AF488-labeled mAbs with 2 × 10^5 of TIM-3 overexpressing FCHO cells. PE-stained cells were analyzed on FACS BD Fortessa. The binding capacity of anti-TIM-3 IgGs was measured by ELISA. AF488-labeled mAbs with 2 × 10^5 of TIM-3 overexpressing FCHO cells. PE-stained cells were analyzed on FACS BD Fortessa.

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PBMC pellets were resuspended in 95% PBS and stained to measure cell-surface expression of CD69. Activated PBMCs were washed seven times with PBS 0.05% Tween20. Fifty microliters of horse serum peroxidase (HRP)-conjugated goat anti-human IgG Fab antibody (Invitrogen) was added to each well and incubated at room temperature for 45 min. The plate was then washed three times with PBS 0.05% Tween20. Finally, ABTS substrate (Invitrogen) was added to each well for colorimetric development for 15 min. The absorbance was read at 405 nm with a plate reader.

For polyclonal phage ELISA, 1 × 10^12 phages from each round of panning were added to culture plates, incubated at 37 °C for 1.5 h. After washing three times, HRP-conjugated anti-M13-polycyclonal antibody was added, and the plates were incubated at 37 °C for 45 min. After three washes with PBST, ABTS was added for color development. The absorbance at 405 nm was determined with a plate reader.

Mixed lymphocyte reaction assay
Dendritic cells (DCs) were generated by cultivating monocytes isolated from PBMCs using a monocyte purification kit (Miltenyi Biotec) in vitro for 7 days with 500 units/mL interleukin-4 and 250 units/mL GM-CSF (R&D Systems). CD4^+ T cells (1 × 10^5) cells) and allogeneic DCs (1 × 10^5 cells) were co-cultured with different concentrations of antibodies. After 5 days, interferon gamma (IFN-γ) secretion in culture supernatants was analyzed using ELISA (R&D Biosciences).

Induction of CD69 expression by m101
Each reaction was set up in complete culture media (RPMI-1640, 10% FBS, penicillin-streptomycin) with 1 × 10^5 PBMCs, 0.25 µg/mL of anti-CD3 mAb (Mouse Anti-Human CD3, BD Biosciences), 0.2-µg/mL of anti-CD28 mAb (Mouse Anti-Human CD28, BD Biosciences), and the tested antibody at the desired final concentration. The complete reaction was incubated for 48 h at 37 °C, then cells were collected by centrifugation (400 × g for 5 min) and stained to measure cell-surface expression of CD69. Activated PBMC pellets were resuspended in 95 µL of FACS buffer (PBS, 1% BSA, 0.1% NaCl) and 5 µL of FITC-labeled CD69 antibody (BioLegend FN50, 100-µg/mL), followed by a 30 min incubation on ice. Labeled cells were washed twice with fresh FACS buffer by pelleting (400 × g for 5 min) and resuspension, and final cell pellets were resuspended in FACS buffer at 1 × 10^6 cells/mL. Staining was assessed by flow cytometry (Thermo Fisher Scientific, Attune NxT) and the results were analyzed using Flowjo and GraphPad Prism V6 software.

Antibody-induced internalization assay
TIM-3-overexpressing FCHO cells were seeded on six-well chamber slides (1 × 10^5 cells per well) and cultured for 24 h prior to treatment and subsequent immunostaining. Cells were then incubated with m101, LY3321367, or irrelevant IgG1 coincubated with 100 nM for 1.5 h at 4 °C. After 1.5 h of incubation at 4 °C, the inoculum was removed and replaced with complete growth medium, and further incubated for 4 h at 37 °C. After the incubation period, unbound antibody was washed off with PBS and cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then permeabilized in PBS plus 0.2% Triton-X-100 for 10 min, and nonspecific labeling was blocked in PBS plus 5% BSA. Antibodies that were bound to the cell surface and antibodies that had been internalized were visualized by incubating cells with Alexa Fluor 647-labeled goat anti-human IgG (Thermo Fisher Scientific). Nuclei were visualized with DAPI. As a control, cells were fixed after the 4 °C incubation (T0). Coverslips were mounted using ProLong Gold Antifade reagent (Thermo Fisher Scientific) for imaging. High-resolution laser confocal image sections were acquired using a Leica TCS SP8 03040108.

Surface plasmon resonance assay
The binding kinetic of m101 to TIM-3-Fc protein was measured using the Octet RED 96 system (Fortebio) at 37 °C. The biotin-labeled TIM-3-Fc was loaded onto SA biosensors. The antibody m101, at concentration ranging from 50 to 3.125 nM was bound to TIM-3-Fc. The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces changes in the thickness of the biological layer, which are measured in real time. Then, the data were fitted to a 1:1 binding model using Octet software.

In vivo studies
To evaluate the antitumor effect of mAb m101 in vivo, a syngeneic model of mouse colon cancer was prepared by inoculating MC38 cells (2 × 10^5 cells) subcutaneously into the right flank of 6- to 8-week TIM-3-humanized C57BL/6N mice. Antibody with a dose of 10-mg/kg or PBS, as negative control, was administered intraperitoneally from the day of tumor inoculation (d 0), and every 3 days afterwards over a total of six doses. Tumor volume was calculated using the equation 0.5 L × W^2, where L and W refer to the length and width of the tumor, respectively.

A xenograft tumor model was also established to evaluate the antitumor effect of m101. NSG mice were inoculated subcutaneously with 1 × 10^6 human lymphoma cells Raji on the right flank at d 0. At d 7, NSG mice were injected with 1 × 10^5 PBMCs intravenously. Then, the mice were treated intraperitoneally with 10 mg/kg m101, 10 mg/kg LY3321367, or PBS twice a week starting on d 8, and over a total of six doses. Tumor volume was monitored twice weekly as described above.

Statistical analyses
Statistical analyses were performed by using GraphPad Prism V6 software. Data are expressed as means ± SEM from three independent experiments. Two group comparisons were performed by unpaired Student’s t test. Multiple group comparisons were performed by one-way ANOVA followed by the Dunnett’s t test. The level of statistical significance was set at *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS
Screening of phage and mammalian cell libraries against TIM-3
To generate high-affinity mAbs against TIM-3, we developed a novel antibody screening platform that combines phage-based panning and the mammalian cell-based screening (Fig. 1a). We constructed a fully human phage display antibody library from genes of PBMCs, and a heavy chain replacement library was constructed by FACS screening, the two cell populations of the antibody were monitored twice weekly as described above.

Antibody-expressing FCHO cells
For polyclonal phage ELISA (Fig.1c), after first step, consisting of two rounds of phage-based panning (Fig. 1b), was performed against a TIM-3-Fc protein, so a pool of positive clones were selected from the libraries (Fig.1d-f). Analysis...
by sequence alignment revealed that eight clones had unique sequences. These clones were expressed and purified in 293F cells (Fig. 1g), and finally, a panel of mAbs was identified and characterized based on their binding activity to TIM-3 antigen. Among these, the m101 clone exhibited the best binding ability to TIM-3 antigen. As shown in Fig. 1h, m101 bound to TIM-3-Fc in a dose-dependent manner and with a half maximal effective concentration (EC50) of 1.26 nM. Thus, subsequent experiments focused on the characterization and functional analysis of m101.

Sequence analysis
To further characterize the VH sequences of m101, we analyzed in detail the recombination frequency of these mAbs to human TIM-3-Fc by ELISA. The results showed that eight mAbs were specific for TIM-3 antigen. Among these, the m101 clone exhibited the best binding ability to TIM-3 antigen. As shown in Fig. 1h, m101 bound to TIM-3-Fc in a dose-dependent manner and with a half maximal effective concentration (EC50) of 1.26 nM. Thus, subsequent experiments focused on the characterization and functional analysis of m101.

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was derived from the IGHV1-3*01 with which it shared 95.83% identity, while the m101 VL gene was derived from IGKV3-20*01, with which it shared 98.58% identity (Fig. 2b). As shown in Fig. 2b, m101 had limited somatic mutations from its germline predecessors, suggesting it possessed a lower level of immunogenicity [3, 14].

m101 enhances T-cell activation in vitro
To further demonstrate the specificity of the m101 in vitro, we performed flow cytometric analysis using 100, 20, 4, or 0.8 nM of m101 directly labeled with AF488 (Fig. 3a). Again, m101 displayed the high binding affinity for TIM-3 expressed by FCHO cells. We further characterized the m101 binding affinity by ForteBio assay. A dose-dependent binding assessment was carried out and relative binding ability values were calculated as described in “Materials and Methods.” Purified m101 was applied to TIM-3-Fc-coated SA sensor (Fig. 3b). According to the results, m101 bound TIM-3-Fc with a dissociation constant ($K_D$) of $5.26 \times 10^{-10}$ (M). Collectively, these results indicated that mAb m101 possesses good binding affinity to TIM-3 in vitro. To evaluate m101 effect on T-cell activation, we set up a mixed lymphocyte reaction assay using human PBMCs from healthy donors, and PBMC activation was measured using IFN-γ release. M101 alone enhanced IFN-γ production compared to the isotype control (Fig. 3c). A control anti-TIM-3 antibody, and LY3321367 developed by Eli Lilly and Company, also resulted in increase of IFN-γ levels in the cell culture supernatant (Fig. 3c). We further intended to specifically examine T-cell responses in vitro in human PBMCs. Consistent with the results of IFN-γ expression, CD69 expression on PBMCs was also upregulated upon addition of m101 and LY3321367 in a dose-dependent manner (Fig. 3d). Previous reports have shown that anti-TIM-3 antibody can induce an internalization effect of TIM-3 [29], which can shut down the entire TIM-3 mediated signaling regardless of the ligands. To confirm whether m101 was internalized upon incubation at 37 °C, we performed confocal microscopy studies. TIM-3 overexpressing FCHO cells were incubated with LY3321367, m101, or isotype control for 1.5 h at 4 °C, then unbound antibody was washed off with PBS and followed by incubation at 37 °C in complete media to start the internalization process. Finally, cells were subsequently fixed and stained with AF647 antihuman IgG. At time point 0, membrane staining was observed (Fig. 3e). After 4 h of incubation at 37 °C, confocal microscopy confirmed that both m101 and LY3321367 were internalized (Fig. 3e). The presence of intracellular antibodies in the cytoplasm of the cells and a decrease in surface expression were observed (Fig. 3e). These results indicated that our novel screening platform could discriminate antibodies with good biological efficacy.

Demonstration of the antitumor efficacy of m101 in two humanized mouse models
The in vivo antitumor activity of m101 was first tested in humanized TIM-3 mice, subcutaneously implanted with the murine colon cancer MC38 cell line. C57BL/6N TIM-3 humanized mice were inoculated subcutaneously with MC38 cells on 0 and subsequently treated by intraperitoneal injection of mAb m101.
Tumor growth was assessed from d 7 to d 25. As shown in Fig. 4b, treatment of tumor-bearing mice with m101 (10 mg/kg) induced significant and durable tumor regression compared to the mice of the control group treated with PBS, and individual mouse tumor growth is shown in Fig. 4d. However, the results of the last tumor measurement on d 25 showed that there was no significant difference in tumor size between the two groups (Fig. 4c).

To further evaluate the therapeutic activity of m101, we chose a human xenograft NSG mouse model inoculated with PBMCs as an in vivo model (Fig. 5a). The therapeutic efficacy was compared among groups treated with m101, LY3321367, or control (Fig. 5b–c), and the individual mouse tumor growth is shown in Fig. 5d–f. The tumor growth was significantly inhibited in the m101-treated group compared with the control group (Fig. 5b). The results of the last tumor measurement showed a significant difference in tumor size between control and m101-treated mice, while treatment with LY3321367 did not induce significant inhibition of tumor growth (Fig. 5b, c). These results indicated that m101 possesses good antitumor activity in vivo.

**DISCUSSION**

The development of therapeutic mAb remains a key issue to fulfill the great needs of biological drugs. Phage display platforms represent powerful in vitro selection techniques for the display and screen of target-specific antibodies. However, it has specific drawbacks, such as inability to display full-length antibody fragment [19, 20] and insufficient affinity for effective clinical use [16]. We sought to resolve this issue by integrating phage and mammalian cell display platforms, and the results suggested that this would overcome the limitations of phage and mammalian cell display platforms and enable effective and reliable selection of full-length IgG antibodies with high binding affinity.

Generating high-affinity antibodies against important drug targets is a critical step in the development of therapeutic mAb

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**Fig. 3** Functional analysis of m101 mAb. **a** m101 binds human TIM-3 expressed on the surface of FCHO cells. The mAb m101 was directly conjugated to PE. The m101 binding specificity was determined by incubating serial dilutions of the AF488-labeled m101 with 2 × 10^5 of TIM-3 overexpressing FCHO cells. **b** Affinity analysis of m101 mAb to TIM-3 measured by BLI in OctetRED96. The TIM-3-Fc protein was immobilized on activated SA biosensors. The analytes consisted of serial dilution (between 50 and 3.125 nM) of m101 mAb. Binding kinetics was evaluated using a 1:1 Langmuir binding model by ForteBio Data Analysis 7.0 software. **c** Mixed lymphocyte reaction assay to detect IFN-γ secretion. CD4^+ T cells (1 × 10^5 cells) and allogeneic DCs (1 × 10^5 cells) were co-cultured with different concentrations of antibodies. After 5 days, IFN-γ secretion in culture supernatants was analyzed by ELISA. **d** Induction of CD69 expression. PBMCs were activated by anti-CD3 and anti-CD28 antibodies and incubated with the tested antibody at the desired final concentration (25, 50, 100 nM) for 48 h at 37 °C. Then cells were collected and stained by FITC-labeled CD69 antibody. **e** Internalization of anti-TIM-3 mAbs in TIM-3 overexpressing FCHO cells. The cells were permeabilized and stained with AF647 goat antihuman IgG (red) and DAPI (blue).
because high antibody affinity allows for a lower dosage. In this study, we produced anti-TIM-3 antibody with high affinity by integrating phage and mammalian cell display platforms, using phages to display random libraries and mammalian cells to display full-length IgGs. The advantage of this approach is that it reduced the number of irrelevant clones by two rounds of phage display panning and allowed construction of mammalian cell libraries using phage library outputs, which improved the efficiency. In addition, the selection of mammalian cell display allows highly controlled, real-time selection that can enable a finely discriminating clones exhibiting different properties such as affinity and features that can be obtained effectively.

To validate this new technology, we chose TIM-3 as the target. It is a promising candidate for cancer immunotherapy first reported in 2002 [27]. TIM-3 is expressed on IFN-γ-producing T cells, FoxP3+ Treg cells, and innate immune cells (macrophages and DCs) [27]. The expression of TIM-3 on these cells is required for the maintenance of immunosuppressive environments [30]. Previous data collected in multiple preclinical cancer models indicated that anti-TIM-3 treatment improves T-cell function [31]. Therefore, there is a great significance for the development of antihuman TIM-3 therapeutic antibodies.

Using this strategy, we obtained a fully human m101 antibody with high affinity, binding human TIM-3-Fc with a dissociation constant ($K_d$) of $5.26 \times 10^{-10}$ (M). All the results of in vitro assay indicated that our novel screening platform could identify antibodies with good biological efficacy. Compared to antibodies, which was induced by high-affinity mutations, germline-like antibodies have a lower level of immunogenicity. Sequence analysis confirmed that m101 is a germline-like antibody with limited mutations, and thus exhibited a lower level of immunogenicity. Taken together, those results can identify germline-like antibodies with high affinity and low immunogenicity using our new platform.

TIM-3 has been reported to have multiple ligands [32–34]. It is becoming increasingly clear that blocking the interactions between TIM-3 and its ligands can enhance antitumor responses [35–37]. It is important that the anti-TIM-3 antibody be able to block these ligand-TIM-3 interactions to prevent an inhibitory signal from TIM-3 [36, 37]. We met this goal by confirmation of the internalization effect of m101, for internalization effect is considered as a completely block effect of TIM-3 signal. Two different in vitro experimental scenarios indicated T lymphocyte activation upon m101 stimulation, and humanized mouse tumor models showed that m101 enhanced antitumor immunity and suppressed tumor growth. Those results showed that m101 can serve as effective cancer therapeutic antibody. Further assessment of m101 is needed to establish the details underlying its mechanism of action.

In conclusion, we created a new full-length antibody screening and selecting strategy. For this strategy, the combination of phage display biopanning step of enrichment for polyclones with binding affinity and two FACS steps of mammalian cell library screening allowed efficient and rapid discovery of IgGs. Using this platform, we identified and characterized a fully human germline-like anti-TIM-3 IgG antibody named m101 with detailed
characterization of its in vitro and in vivo efficacy, indicating the complete feasibility of the new strategy. To our knowledge, this is the first report describing a combined phage/mammalian cell display platform, which we believe can be easily applicable for the identification of clinical candidate antibodies.

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AUTHOR CONTRIBUTIONS
YLW, CT, and TLY designed all the experiments. YJJ, DY, XLT, YK, LZ, CL, ZLY, HXL, XCZ, and WZ mainly conducted the in vitro and in vivo experiments. HL constructed the mammalian cell library and screened and purified the IgGs. TLY, YJJ, and YLW composed the manuscript. All authors contributed to the article and approved the submitted version.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

REFERENCES
1. Grilo AL, Mantalaris A. The increasingly human and profitable monoclonal antibody market. Trends Biotechnol. 2019;37:9–16.
2. Kumar R, Paray HA, Shivastava T, Sinha S, Luthra K. Phage display antibody libraries: a robust approach for generation of recombinant human monoclonal antibodies. Int J Biol Macromol. 2019;135:907–18.
3. Hu D, Zhu Z, Li S, Deng Y, Wu Y, Zhang N, et al. A broadly neutralizing germline-like human monoclonal antibody against dengue virus envelope domain III. PLoS Pathog. 2019;15:e1007836.
4. Tabasinezhad M, Talebkhan Y, Wenzel W, Rahimi H, Omidinia E, Mahboudi F. Trends in therapeutic antibody affinity maturation: from in-vitro towards next-generation sequencing approaches. Immunol Lett. 2019;212:106–13.
5. Frenzel A, Schirrmann T, Hust M. Phage display-derived human antibodies in clinical development and therapy. mAbs. 2016;8:1177–94.
6. Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD. Isolating and engineering human antibodies using yeast surface display. Nat Protoc. 2006;1:755–68.
7. Zhou C, Jacobsen FW, Cai L, Chen Q, Shen D. Development of a novel mammalian cell surface antibody display platform. mAbs. 2010;2:508–18.
8. Li F, Liu YH, Li YW, Li YH, Xie PL, Ju Q, et al. Construction and development of a mammalian cell-based full-length antibody display library for targeting hepatocellular carcinoma. Appl Microbiol Biotechnol. 2012;96:1233–41.
9. Barbas CF 3rd, Kang AS, Lerner RA, Benkovic SJ. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci U S A. 1991;88:7978–82.
10. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. Annu Rev Immunol. 1994;12:433–55.
11. Schofeld DJ, Pope AR, Clementel V, Buckell J, Chapple S, Clarke KF, et al. Application of phage display to high throughput antibody generation and characterization. Genome Biol. 2007;8:R254.
12. Kügler J, Wilke S, Meier D, Tomszak F, Frenzel A, Schirmann T, et al. Generation and analysis of the improved human HAL9/10 antibody phage display libraries. BMC Biotechnol. 2015;15:10.
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13. Schwimmer LJ, Huang B, Giang H, Cotter RL, Chemla-Voggel DS, Dy FV, et al. Discovery of diverse and functional antibodies from large human repertoire antibody libraries. J Immunol Methods. 2013;391:60–71.

14. Ying T, Du L, Ju TW, Prabakaran P, Lau CC, Lu L, et al. Exceptionally potent neutralization of Middle East respiratory syndrome coronavirus by human monoclonal antibodies. J Virol. 2014;88:7796–805.

15. Jung S, Honegger A, Plückthun A. Selection for improved protein stability by phage display. J Mol Biol. 1999;294:163–80.

16. Coia G, Hudson PJ, Irving RA. Protein affinity maturation in vivo using E. coli mutator cells. J Immunol Methods. 2001;251:187–93.

17. Low NM, Holliger PH, Winter G. Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. J Mol Biol. 1996;260:359–68.

18. Schütte M, Thullier P, Pelat T, Wezler X, Rosenstock P, Hinz D, et al. Identification of a putative Crf splice variant and generation of recombinant antibodies for the specific detection of Aspergillus fumigatus. PLoS One. 2009;4:e6625.

19. Hust M, Maiss E, Jacobsen HJ, Reinard T. The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease. J Virol Methods. 2002;106:225–33.

20. Moghaddam A, Borgen T, Stacy J, Kausmally L, Simonsen B, Marvik OJ, et al. Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monacetylmorphine but not morphine. J Immunol Methods. 2003;280:139–55.

21. Akamatsu Y, Pakabunto K, Xu Z, Zhang Y, Tsurushita N. Whole IgG surface display on mammalian cells: Application to isolation of neutralizing chicken monoclonal anti-IL-12 antibodies. J Immunol Methods. 2007;327:40–52.

22. Ellmark P, Ohlin M, Borrebaeck CA, Furebring C. A novel mammalian display system for the selection of protein-protein interactions by decoy receptor engagement. J Mol Recognit. 2004;17:316–22.

23. Tomimatsu K, Matsumoto SE, Tanaka H, Yamashita M, Nakanishi H, Teruya K, et al. A rapid screening and production method using a novel mammalian cell display to isolate human monoclonal antibodies. Biochim Biophys Res Commun. 2013;441:59–64.

24. Zhou C, Shen WD. Mammalian cell surface display of full length IgG. Methods Mol Biol. 2012;907:293–302.

25. Romero D. Immunotherapy: PD-1 says goodbye, TIM-3 says hello. Nat Rev Clin Oncol. 2016;13:202–3.

26. Anderson AC. Tim-3: an emerging target in the cancer immunotherapy landscape. Cancer Immunol Res. 2014;2:393–8.

27. Monney L, Sabatos CA, Gaggiol Jl, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature. 2002;415:536–41.

28. Yu F, Song H, Wu Y, Chang SY, Wang L, Li W, et al. A potent germline-like human monoclonal antibody targets a pH-sensitive epitope on H7N9 influenza hemagglutinin. Cell Host Microbe. 2017;22:471–83.e5.

29. Kuang Z, Li L, Zhang P, Chen B, Wu M, Ni H, et al. A novel antibody targeting TIM-3 resulting in receptor internalization for cancer immunotherapy. Antib Ther. 2020;3:227–36.

30. Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, et al. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. Blood. 2009;113:3821–30.

31. Kim JE, Patel MA, Mangraviti A, Kim ES, Theodros D, Velarde E, et al. Combination therapy with anti-PD-1, anti-TIM-3, and focal radiation results in regression of murine gliomas. Clin Cancer Res. 2017;23:124–36.

32. Zhu C, Anderson AC, Schubart A, Xiong H, Imitol J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol. 2005;6:1245–52.

33. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol. 2012;3:832–42.

34. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. Nature. 2015;517:386–90.

35. Yang R, Sun L, Li CF, Wang YH, Yao J, Li H, et al. Galectin-9 interacts with PD-1 and TIM-3 to regulate T cell death and is a target for cancer immunotherapy. Nat Commun. 2021;12:832.

36. Sabatos-Peyton CA, Nevin J, Brock A, Venable JD, Tan DJ, Kassam N, et al. Blockade of Tim-3 binding to phosphatidylinerine and CEACAM1 is a shared feature of anti-Tim-3 antibodies that have functional efficacy. Oncoimmunology. 2018;7:e1385690.

37. Kayama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. Nat Commun. 2016;7:10501.