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Developing Cell-Specific Antibodies to Endothelial Progenitor Cells Using Avian Immune Phage Display Technology

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This study aims at generating immune chicken phage display libraries and single-chain antibodies (scFvs) specifically directed against cell surface markers of cultured peripheral blood mononuclear cells (PBMCs) that contain endothelial progenitor cells (EPCs). In contrast to previous approaches that use well-defined recombinant antigens attached to plastic surfaces that may alter the structure of the proteins, the authors describe a method that maintains the cell surface markers on live cells while providing the opportunity to rapidly screen entire libraries for antibodies that bind to unknown cell surface markers of progenitor/stem cells. Chickens immunized with live EPCs, consisting of a heterogeneous population of lymphocytes and monocytes, demonstrated a robust immune response. After three rounds of biopanning, the authors purified and characterized three unique scFvs called UG1-3. Codon-optimized recombinant UG1 (gUG-1) shows binding by flow cytometry to circulating CD14-positive cells in peripheral blood consistent with predominant expression of a target protein on monocyte subsets. The authors describe the successful use of immunization of chickens for the generation of scFvs against a heterogeneous population of EPCs displaying unknown cell surface markers and demonstrate the strong potential of phage display technology in the development of reagents for the isolation and characterization of stem/progenitor cells. (*Journal of Biomolecular Screening*. 2011;16:744–754)

Key words: phage display, single-chain antibodies, endothelial progenitor cells, cell surface markers, vasculature

INTRODUCTION

THERE IS CONSIDERABLE INTEREST in the use of hematopoietic stem cells or other cells with angiogenic potential such as endothelial progenitor cells (EPCs) in the treatment of ischemia, restenosis, diabetic vasculopathy, and occlusive vascular disease, which includes coronary artery disease, cerebrovascular disease, and peripheral artery disease.^{1–6}

The discovery of EPCs introduced a novel concept stating that vasculogenesis can occur postnatally, whereby, in response to various stimuli (especially ischemia), EPCs home, differentiate, proliferate, and incorporate into resident endothelial cells.⁷ EPCs are commonly defined as a heterogeneous, fibronectin-adherent

mononuclear cell population derived from bone marrow, umbilical cord, or peripheral blood⁸ and have ever since been cultured using methods that are associated with cells of distinct phenotypes: colony-forming unit Hill cells (CFU-Hills), circulating angiogenic cells (CACs), and endothelial colony-forming cells (ECFCs).⁹ The CFU-Hills or CACs were also termed *early outgrowth cells* (early EPCs) because they appear in culture after 4 to 9 days, whereas ECFCs are often referred to as *late outgrowth cells* (late EPCs) due to their appearance in culture after 1 to 3 weeks.⁹ Early EPCs do not form tubules in vitro⁸ but adhere to mature endothelial cells and exert their function via paracrine effects, whereas late EPCs are capable of in vitro tubule formation without the presence of mature endothelial cells.^{8–10} All EPCs take up acetylated low-density lipoprotein (acLDL) and Ulex europeus agglutinin I (UEA-1) and share the expression of cell surface markers such as vascular endothelial growth factor receptor-2 (VEGFR2), CD31, von Willebrand factor (vWF), and endothelial nitric oxide synthase (eNOS), whereas they differ in the expression of other cell surface markers (late outgrowth are CD133⁺, CD115⁺, CD45⁺, CD14⁺, CD34⁺ and early outgrowth are CD133⁺ and CD115⁺) with conflicting reports on the expression of some early EPC markers (CD45^{+/–}, CD14^{+/–}, CD34^{+/–}).^{7–15}

Research in this area is hindered, however, by the absence of an EPC marker, and there is a clear need to develop novel assays and highly specific reagents.

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Murine monoclonal antibody (MAb) technology has found wide acceptance but suffers from limitations such as randomly identified antibodies against immobilized antigens or the absence of a robust rodent immune response against proteins that are highly conserved in closely related species.

We sought an alternative system for generating EPC-specific MAbs. Phage display has become a powerful technology for the generation of high-affinity reagents to study angiogenesis.¹⁶ Chickens have become effective hosts for generating high-specificity recombinant antibodies to mammalian proteins using phage display, as they can circumvent many of the common problems encountered with murine immunizations.^{17,18} Most importantly, however, the amino acid homology between the mammalian and avian orthologues of a given protein is typically low, and some mammalian proteins may not even exist in avians.¹⁹ The immunoglobulin response of chickens to highly conserved mammalian proteins is therefore reliably robust, often exhibits high affinity, and potentially targets a broad spectrum of epitopes on protein immunogens.^{17,20,21}

In this study, we aimed at applying phage selections to *in vitro* cultured, intact EPCs displaying the full repertoire of potential cell surface markers and set out to generate antibodies to heterogeneous proangiogenic endothelial progenitor cell preparations that contain mainly lymphocytes and monocytes using chicken immune phage display technology. The ultimate goal was to develop single-chain antibodies that bind to the surface of live EPCs and internalize. These scFvs could be used not only as reagents to detect and enrich cells prior to applications in regenerative medicine such as diabetic wound healing or cardiac repair after acute myocardial infarction but also preferably as tools that aid in the specific targeting of gene delivery systems to circulating angiogenic cells to enhance their therapeutic potential. We identified and characterized three novel scFvs, termed UG 1-3. Codon-optimized UG1, called gUG1, was obtained in higher yields and binds predominantly to monocyte subsets in peripheral blood. The strategy outlined here can be applied to generate specific antibodies against other mammalian cells that are of therapeutic interest such as embryonic stem cells or human mesenchymal stem cells and will greatly aid in the clinical translation of well-characterized, cell-based therapeutics in regenerative medicine applications.

MATERIALS AND METHODS

EPC isolation and characterization

This study was approved by the Clinical Research Ethics Committee, University College Hospital, Galway, Ireland. Peripheral blood was obtained from donors with informed consent. EPCs were cultured as previously described.²² Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation (Ficollpaque, GE Healthcare, Uppsala, Sweden). After purification with three washing steps, 10×10^6

PBMCs were plated on fibronectin-coated, 6-well plates and cultured in endothelial cell basal medium-2 (Clonetics, Lonza, Walkersville, MD) supplemented with EGM-2 single aliquots (Clonetics, Lonza) consisting of fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, heparin, gentamicin/amphotericin-B, and ascorbic acid. After 4 days in culture at 37 °C and 5% CO₂, adherent cells were stained for the uptake of diI-labeled acLDL (Molecular Probes, Carlsbad, CA) and the binding of fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin I (Sigma, St. Louis, MO). For further characterization, we examined the EPCs with a 1:50 sheep anti-human vWF-FITC antibody and with a 1:200 rabbit anti-human VEGFR2 in combination with a 1:100 dilution of a goat anti-rabbit antibody (all from Abcam, Cambridge, MA). Images were acquired at room temperature using an Olympus DP70 color camera (Tokyo, Japan) with Olympus Biosystems AnalySISD software. EPCs were cultured for 7 days, nonadherent cells removed by phosphate-buffered saline (PBS) wash, the adherent cells detached with 5 mM EDTA and frozen down at -80 °C in a 10% DMSO/90% fetal bovine serum solution.

Chicken immunization

Two ISA brown chickens were supplied by and immunizations conducted at Agro-Bio (La Ferté, St. Aubin, France). Cryopreserved fibronectin adherent cells were thawed and centrifuged at 400g for 5 min room temperature (RT). The freezing medium was removed and cells resuspended in a total of 10 mL of supplemented medium and incubated at 37 °C for 45 min. Cells were washed twice with sterile PBS solution. Both chickens were injected intradermally (under the wing) with 5×10^5 cells in a total volume of 200 µL PBS at day 0, 21, and 42. Chickens were prebled at day 0 and bled again at day 49. At day 49, the chickens were sacrificed and the spleen and bone marrow were harvested, homogenized, and stored in TRIzol reagent (Invitrogen, Carlsbad, CA).

Avian scFv library generation

ScFv library generation was performed as described previously.^{18,23} Briefly, RNA was extracted from spleen and bone marrow samples using TRIzol reagent, and first-strand cDNA was synthesis performed using the Superscript First Strand synthesis system for RT-PCR (Invitrogen). Antibody V_H and V_L genes were amplified using the primers CSCVHo-F (5'-GGTCAGTCCTCTAGATCTTCGCGCGTGACGTTG GACG AG-3') and CSCG-B (5'-CTGGCCGGCCTGGCCACT AGTGGAGGAGAC GATGACTTCGGTCC 3') to amplify the chicken V_H domains. Primers CSCVK (5'-GTGGCCCAGGCG GCCCTG ACCTAGGACGGTCAGG 3') and CKJo-B (5'-GGA AGATCTAG AGGACTGACC TAGGACGGT CAG-3') were used to amplify the V_L domains. The single-chain antibody library

was assembled in a V_L - V_H scFv format, with a flexible linker (peptide sequence GGSSRSS) by SOE-PCR using the pooled V_H and V_L PCR products and subcloned into the pComb3X vector (Scripps Institute, La Jolla, CA). Electrocompetent XL1 Blue bacteria (Stratagene, La Jolla, CA) were mixed with the library prior to electroporation with a Micropulser (Bio-Rad, Hercules, CA). The bacteria were incubated at 37 °C at 220 rpm for 1 h. Ampicillin (25 µg/mL) and tetracycline (10 µg/mL) were added for 1 h. After transfer to 183 mL terrific broth containing ampicillin and tetracycline, 2 mL of VCSM13 helper phage was added and the samples were incubated for 2 h at 37 °C, 220 rpm. Kanamycin was added at 25 µg/mL, and all cultures were incubated for 6 h at 37 °C and 220 rpm. The bacteria were spun down at 3000g for 15 min and phages precipitated from the supernatant by addition of 8 g PEG, 6 g NaCl, followed by an incubation period of 30 min on ice and centrifugation at 15 000g for 15 min at 4 °C. The phage pellet was washed with 1% (w/v) bovine serum albumin (BSA) in PBS and passed through a 0.2-µm filter.

Biopanning of phage libraries on cultured EPCs

We devised the following protocol for selecting and enriching (panning) antibody displaying phage on the surface followed by their uptake into live cells, a process we refer to here as *biopanning of libraries*. The phage library was dialyzed in PBS for 1 h and blocked in 1% BSA, PBS solution for 1 h at 4 °C. The library was added to the supernatant of the cultured EPCs and incubated with gentle shaking at 4 °C for 45 min. The cells were washed once with PBS and once with 100 mM glycine (pH 2.7) to reduce the high numbers of antibodies that bound weakly to the cell surface. The EPCs were incubated at 37 °C for 1 h to allow for internalization of antibody-displaying phage. Cells were washed once with PBS, trypsinized for 10 min (37 °C, 5% CO₂), washed with serum containing medium, and spun down at 1500 rpm for 5 min. All cells were lysed with 100 mM TEA, neutralized with 2M Tris (pH 7.2) and added to 5 mL of XL1 Blue for 1 h to allow phage reinfection. The bacteria were plated on superbroth/ampicillin plates and incubated overnight at 37 °C. Two further rounds of phage preparations and panning of phage libraries were performed using cultured EPCs as screening targets.

Isolation and purification of novel scFvs

The library was transformed into XL1 blue after three rounds of biopanning as described above. Clones were randomly picked from a plate and the antibody amplified by PCR using primers ompseq (5'-AAGACAGCTATCGCGATTGCAG-3') and gback (5'-GCCCCCTTA TTAGCGTTT GCCATC-3'). PCR products were digested with *Alu* I for 4 h at 37 °C and analyzed on a 4% agarose gel for unique restriction digest patterns. All unique antibody clones from the third round of biopanning were submitted

to MWG (Germany) for sequencing analysis using primers ompseq and gback. Clones were grown in 2 mL terrific broth (TB), 50 µg/mL carbenicillin, 1 mM MgCl₂ at 37 °C, 220 rpm overnight, transferred to 250 mL of fresh TB/carbenicillin, 1 mM MgCl₂, 1% glucose, and incubated at 37 °C, 250 rpm for 8 h. The cultures were centrifuged at 2500g at 4 °C for 15 min, the pellets were resuspended in 250 mL TB, 50 µg/mL carbenicillin, 1 mM MgCl₂, and induction of antibody expression was initiated by the addition of 0.5 mM IPTG. After incubation at 30 °C, 250 rpm for 24 h, the bacteria were spun down at 6000g for 15 min. The soluble, secreted antibody containing a tag composed of six histidines was isolated from the filter-sterilized bacterial supernatant by Ni-NTA agarose column purification (Qiagen, Venlo, the Netherlands) via FPLC (GE Healthcare). An Ni-NTA column (Qiagen) was equilibrated with 10 mM imidazole, 0.5 M NaCl in PBS (pH 7.4); the antibody sample was applied and eluted in a stepwise gradient at 80% and then 100% of a 1 M imidazole elution buffer (1 M imidazole, 0.5 M NaCl in PBS, pH 7.4). Eluted fractions of the antibodies were either dialyzed against PBS or desalted using FPLC gel permeation chromatography (Superdex 200 10/300 gel filtration column, GE Healthcare).

Characterization of antibodies

Flow cytometry. The chicken immune response after injection of EPCs was determined by FACS (flow cytometry). EPCs were harvested by cell scraping, incubated with 1:200 dilutions of serum from chicken bleeds with FACS buffer (1% BSA, 0.02% sodium azide in PBS) and incubated on ice for 30 min. Cells were washed twice with FACS buffer and incubated with a 1:100 dilution of rabbit anti-IgY FITC (Pierce, Rockford, IL) preincubated in FACS buffer containing 20% human serum to reduce the background binding of the anti-IgY antibody. Cells were washed and resuspended in 200 µL of FACS buffer plus 20 µL of 30 µM 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) for dead cell exclusion.

Surface marker characterization of EPC cultures before and after cryopreservation and binding of scFvs to PBMCs was examined by flow cytometry. EPC cultures, cryopreserved EPCs, and PBMCs were prepared as described above, washed, and resuspended in FACS buffer (PBS, 2% FCS, 0.05% NaN₃, pH 7.4) in the presence of human FcR blocking reagent (Miltenyi Biotec, Germany) at 4 °C for 10 min. Cells were again washed and resuspended in FACS buffer at 5×10^6 cells/mL and transferred into FACS tubes (Fisher Scientific, Dublin, Ireland) at 100 µL/tube. EPCs were incubated at 4 °C for 20 min with three to four color combinations of the following monoclonal antibodies: mouse anti-human CD45-PE (clone 5B1; Miltenyi Biotec), mouse anti-human CD3-AF488 (clone UCHT1), mouse anti-human CD14-PE-Cy7 (clone M5E2), mouse anti-human CD19-PE (clone HIB19), mouse anti-human CD56-APC (clone B159), and mouse anti-human CD34-APC (clone 581/CD34; all from BD

Biosciences, San Jose, CA); mouse anti-human Tie-2/TEK/CD202b-APC (clone 83715; R&D Systems, Minneapolis, MN), and mouse anti-human CD309 (VEGFR-2/KDR; clone ES8-20E6, Miltenyi Biotec). PBMCs were first incubated with and without a previously optimized amount (2.5 μ g) of purified HA-tagged gUG1 at 4 °C for 30 min and then were washed in FACS buffer followed by addition of mouse anti-HA-PE (Miltenyi Biotec) and two to three color combinations of antibodies directed against CD3, CD14, CD19, CD56, CD 34, Tie-2, and CD309 as described above for 20 min at 4 °C. Following all staining steps, cells were washed and resuspended in FACS buffer; the dead cell stain, SYTOX Blue (Invitrogen, Molecular Probes), was added, and the cells were immediately analyzed on a BD Biosciences FACS Aria II flow cytometer. Between 10^5 and 5×10^5 events were acquired for each individual sample. Flow cytometry data were analyzed using FlowJo software (TreeStar Inc., Olten, Switzerland). For all analyses, nonviable (SYTOX-positive) cells and cell aggregates were excluded, and appropriate negative staining controls (fluorescent minus one and/or matched isotype controls) were used to distinguish positive and negative staining populations.

Immunolabeling of cells in vitro using quantum dots

Cells grown on slides were washed in PBS, fixed in 2% PFA for 1 h at RT, washed again with PBS, and blocked in 2% BSA/PBS for 30 min at RT. Immunolabeling of heterogenous EPCs mainly consisting of CD14⁺ monocytes and CD3⁺ T lymphocytes was performed by incubating the cells with a 1:300 dilution of mouse anti-CD3 (eBioscience) or mouse anti-CD14 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 2% BSA/PBS for 1 h at RT. Cells were washed with PBS twice for 5 min and then incubated with a 1:200 dilution of biotinylated, horse anti-mouse IgG antibody in 2% BSA/5% horse serum/PBS for 30 min at RT. Slides were washed twice with PBS for 5 min and incubated with a 1:100 streptavidin Qdot 605 solution (Invitrogen) in 2% BSA/PBS for 1 h at RT. Slides were washed with PBS, stained with a 1:1000 dilution of Hoechst dye for 10 min at RT, washed twice with PBS, mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA), and dried overnight at 4 °C. Images were acquired with a Zeiss LSM510 META confocal microscope as previously described.²⁴

Matrigel tubule assay

Matrigel tubule assay was performed as previously described with modifications.²² Briefly, matrigel (BD Biosciences) was thawed and placed in 4-well glass slides or in 12-well plates at RT for 30 min to allow solidification. DiI- and FITC-labeled EPCs (2×10^4 and 4×10^4 , respectively) were co-plated with 4×10^4 human umbilical vein endothelial cells (HUVECs) and incubated at 37 °C. Tubule formation was defined as a structure exhibiting a length four times its width, and the number of tubules was counted per field under the microscope. Tubule count was

performed for three fields per well in triplicate. The data shown are representative of several similarly performed experiments that consistently gave comparable results.

Codon optimization

Clonal sequence of UG1-3 obtained from MWG (Germany) were submitted to GENEART (Germany) for codon optimization in *Escherichia coli*. The codon-optimized clone (gUG1) was ligated into pComb3X for expression.

UG1 anti-EPC scFv accession number

EMBL:FN555207

RESULTS

Chicken immunizations

Both chickens were immunized successfully with fibronectin-adherent EPC (Fig. 1a) preparations that, based on single-color immunofluorescence microscopy, consisted of mainly T lymphocytes (Fig. 1b) and monocytes (Fig. 1c). Early EPCs also took up acLDL and UEA-1 (Fig. 1d). These heterogenous cultures of EPCs stained positive for endothelial markers such as vWF (Fig. 1e) and VEGFR2 (Fig. 1f). The angiogenic potential of cultured EPCs was assessed in standard matrigel tubule assays. Cultured EPCs did not form tubules (data not shown). However, the numbers of angiogenic tubules formed by HUVECs in matrigel (Fig. 1g) increased twofold when co-cultured with EPCs (Fig. 1h, j), indicating the retention of functionality of cultured progenitor cells after isolation from peripheral blood of healthy adult donors prior to immunizations of avians. Multicolor flow cytometry was used to more comprehensively analyze the cellular constituents of EPC cultures following cryopreservation and thawing (Fig. 2). This demonstrated a high level of viability, predominant expression of the pan-leukocyte marker CD45, and co-existence of a large, CD14⁺ monocytic population (5%–10%) with a smaller, CD14[–] lymphocytic population (Fig. 2a). Subanalyses of these two populations (Fig. 2b) indicated that the lymphocytic populations consisted primarily of CD3⁺ T cells with lesser proportions of CD19⁺ B cells and CD56⁺ NK cells. The monocytic population contained a subset of CD14⁺/CD3⁺ cells but minimal numbers of cells staining positive for CD19 and CD56. Neither population contained significant numbers of CD34⁺ cells. Similar results were obtained for EPCs from a separate donor and for EPCs analyzed prior to cryopreservation (data not shown). The immunoglobulin response of both chickens was assessed via flow cytometry, by comparing binding of the preimmune and terminal bleed IgY to the cells used for immunization (Fig. 3). Both chickens showed a robust immune response as demonstrated by a shift in fluorescent intensity between the terminal bleed and preimmune serum.

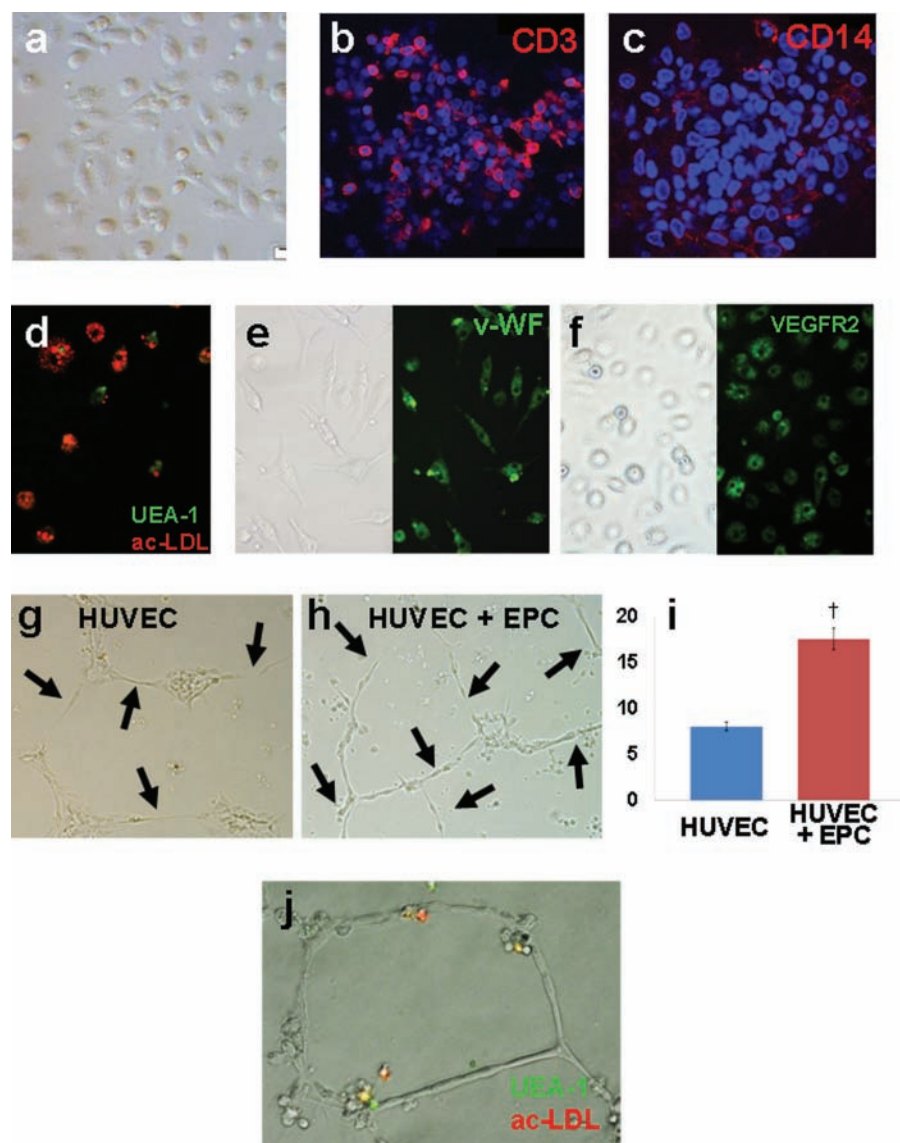


FIG. 1. Characterization of endothelial progenitor cells (EPCs). (a) The immunizing cells are heterogeneous, consisting mainly of (b) T lymphocytes and (c) monocytes, as demonstrated using anti-CD3 and CD14 Qdot 605 streptavidin-conjugated antibodies. EPCs take up (d) fluorescein isothiocyanate (FITC)-labeled UEA-1 (green) and DiI-labeled acLDL (red) and stain positive for (e) FITC-labeled von Willebrand factor or (f) FITC-labeled vascular endothelial growth factor receptor-2 (VEGFR2). Phase contrast pictures (e–f) show the same sections of EPC culture as the fluorescence images. Matrigel assays: Tubule formation of (g) human umbilical vein endothelial cells (HUVECs) and (h) HUVECs co-cultured with EPCs. (i) Augmentation of in vitro tubule formation of HUVECs by EPCs. EPCs alone did not form tubules (data not shown) but (j) demonstrated angiogenic potential in vitro (double stain as in d) as evidenced by their ability to adhere to and enhance tubule formation when co-cultured with HUVECs. White size bars indicate 100 μ m. Matrigel: Phase contrast pictures (g), (h), and (j) show cells at 100 $\times$ magnification.

Library construction

The bone marrow and spleens from both birds were harvested, cDNA was generated, and an scFv library of an estimated titer of 2.7×10^8 clones was successfully constructed. One aliquot of the library was transformed into bacteria, and *Alu* I digest of PCR products was initially performed on 20 randomly

selected colonies to examine the diversity and uniqueness of the antibody clones within the library. A very diverse (>85%) banding pattern was observed after gel electrophoresis after two independent *Alu* I digests (data not shown). We then examined 20 randomly selected colonies obtained after three rounds of biopanning, which led to the identification of three unique scFv banding patterns. These three unique scFvs were

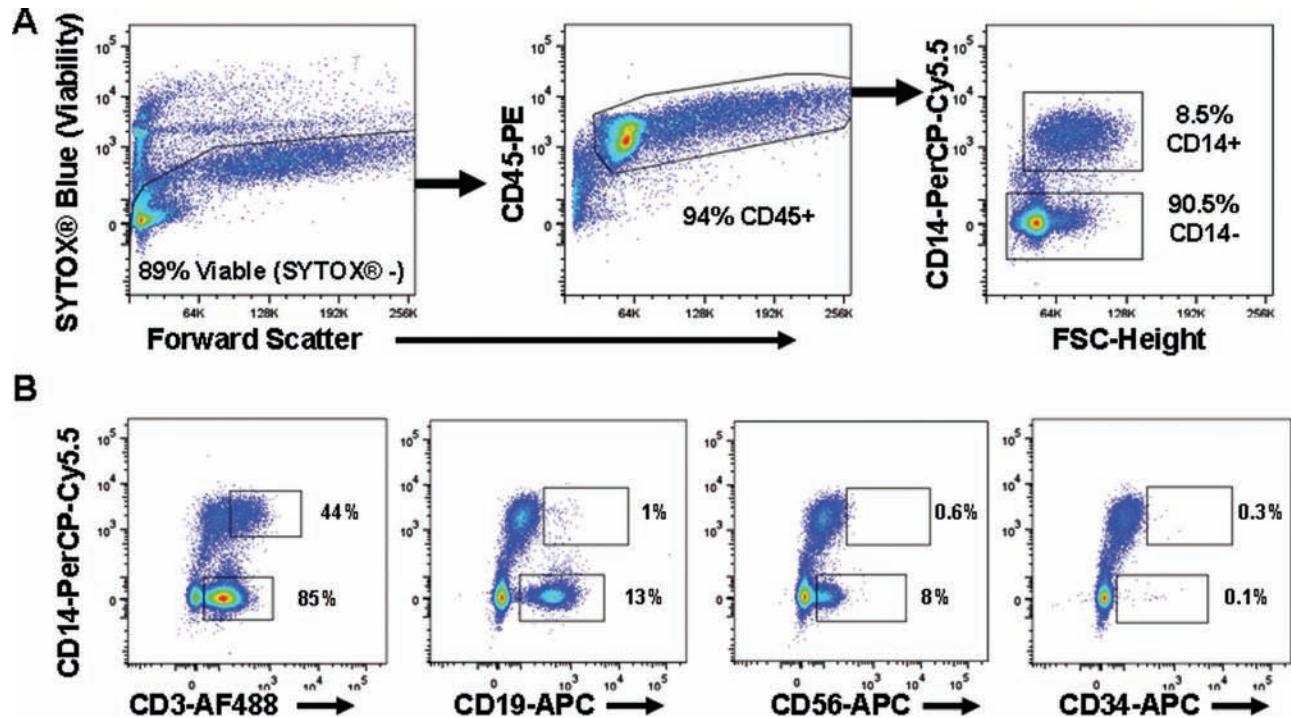


FIG. 2. Multicolor flow cytometric analysis of cryopreserved and thawed endothelial progenitor cell (EPC) culture. (A) Sequential dot plots are shown for viability gating based on exclusion of SYTOX Blue vital dye, surface staining of viable cells for the pan-leukocyte marker CD45, and surface staining of viable, CD45+ cells for the monocyte marker CD14. Relevant proportions are indicated for each defined region. (B) Dot plots are shown for additional surface stains of viable, CD45+ cells within the thawed EPC culture. The percentages with positive staining for each marker are indicated separately for CD14+ and CD14- subsets.

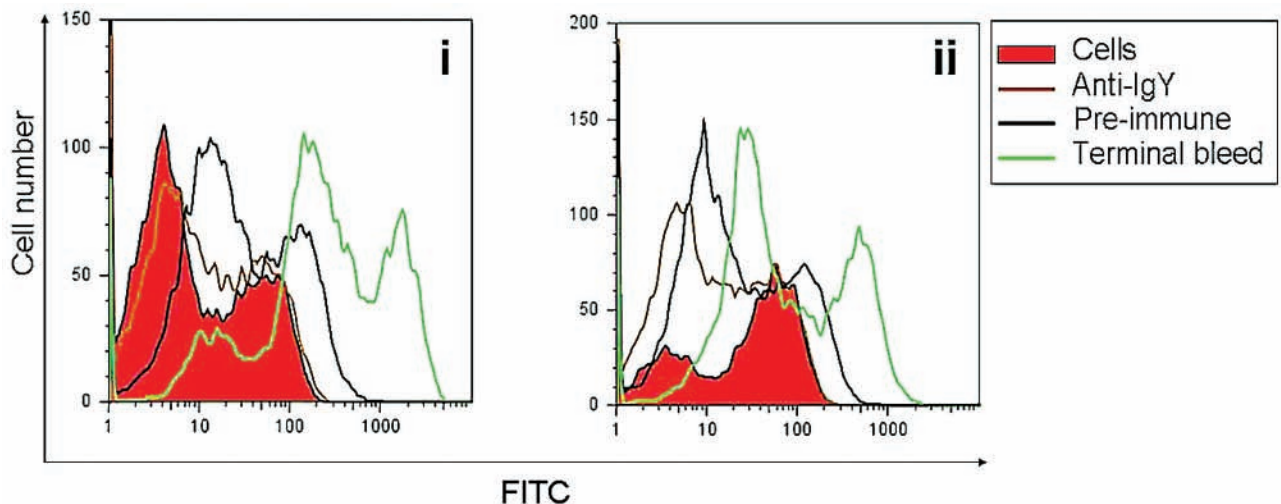


FIG. 3. Cytometric assessment of chicken immune responses using cultured endothelial progenitor cells (EPCs) as targets. Cells were incubated with 1:200 dilution of avian preimmune (black curve) or terminal bleed sera (green curve) and detected with fluorescein isothiocyanate (FITC)-labeled anti-IgY antibody. Negative controls include EPCs stained with the secondary, anti-IgY antibody (brown curve) and EPCs without any antibody stain (red curves). Both chickens (i and ii) showed a good immune response, demonstrating a clear 1-log shift in fluorescent intensity between the terminal bleed (green curve) and preimmune serum (black curve) and a 1- to 2-log shift between the terminal bleed (green curve) and the negative controls (brown or red curves). The two dominant, different populations of cells with their distinct fluorescence intensities (peaks) can be attributed to T lymphocytes (low fluorescence) and monocytes (high fluorescence).

	FR1	+++++ CDR1 +++	FR2	+CDR2+	
germline VL	ALTQPSSVSANPGGTVKITCSG	-----DSSYYGWYQQKAPGSAPVTVIYDNTNRP			50
UG1	ALTQPSSVSANPG ET VKITCSG	----- SS SWYGWYQQKAPGSAPVTVIY SN NQRP			45
UG2	ALTQPSSVSANPG ET VKITCSG	----- GT YRYGW F QQK SP GSALVTVIY W DDERP			45
UG3	ALTQPSSVSAN T ETVKITCSG GG SWYGS YY GWYQQK SP GSALVTVIY ES TKRP				50
	*****.*	*****	.*	***:***:***	*****
	+	FR3	+++ CDR3 +++	FR4	
germline VL	SNIPSRFSGSKSGSTATLTITGVRADDNAVYYCASTDSSSTAGIFGAGTTLTVL				104
UG1	SNIPSRFSGSKSG M GTLTITGV Q VE E AVYY C NE D TS G TA- A FGAGTTLTVL				98
UG2	S GIPSRFSG S A SGSTATLTITGV Q A E DE A MY C GS I DSS- Y VGIFGAGTTLTVL				98
UG3	S DIPSRFSG S L SGST N TLTITGV Q A E DE A V F CGGYDSS S Y VGIFGAGTTLTVL				104
	*,*****	*****	.*	***:***:***	*****
	FR1	CDR1	FR2	+++++CDR2++	
germline VH	AVTLDESGGGLQTPGRALSLVCKASGFTFSSYNMGWVRQAPGKGLEFVAGIDNTGRYTG				60
UG1	AVTLDESGGGLQTPGGALSLV E ASGFTFSS F N M FVVRQAPGKLE W VAGI S GAG S STGY				60
UG2	AVTLDESGGGLQTPGGALSLVCKASGFTFSS A M Q VVRQAPGKLE W VAGI G NGG-- T YY				58
UG3	AVTLDESGGGLQTPGG G LSLV-KASGF S FSS F N I QVVRQAPGKLE W VAGI N AAG S STGY				59
	*****	*****	.*	***:***:***	*****
	+++++++	FR3	+++++ CDR3 +++++	FR4	
germline VH	GS	AVKGRATISRDNQSTVRLQLNNLRAEDTGTYYCAK	-----	AAGIDAWGHGTEVIVSS	115
UG1	G SAVKGRATISRDNQSTVRL Q VNNLRAEDT G I F CAKAAGY G RYAD A GS I DAWGHG T EV----				120
UG2	GA	AVKGRATISRDNQSTVRLQLNNLRAEDT G T F CAKN- A DAGI P DAGE I DAWGHG T EV----			117
UG3	G SAV Q GRATISRDNQSTVRLQL S NLRAEDT G T Y YCA E SSNCAY G CAGE I DAWGHG T EV----				119
	*:**	*****	*****	.*	*****

FIG. 4. Sequence alignment of UG scFvs. Translated scFv sequences were aligned using the European Bioinformatics Institutes ClustalW2 align program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) and compared with the germline chicken H and L chains. All UG scFvs show evidence of distinct B-cell lineages and mutational deviation (in red) compared with the germline sequence, particularly in the complementarity-determining regions (CDRs; Kabat definition). Importantly, there are unique CDR3 heavy chain regions in each antibody. Both UG2 and UG3 also required codon optimization of corrected sequences for protein expression that introduced a tryptophan or arginine (highlighted in green).

designated UG1-3. We repeated this random selection and *Alu* I analysis several times (data not shown) but were unsuccessful in finding additional unique banding patterns indicating a strong enrichment of UG1-3 after three rounds of biopanning. Interestingly, UG1 accounted for more than 75% of all scFvs at the end of the biopanning, which prompted us to focus on advancing UG1 over UG2 and UG3. The PCR products for each of the three UG clones were submitted for sequencing, translated, and aligned with the chicken immunoglobulin germline sequence (Fig. 4). This analysis revealed evidence of three fully independent sequences that do not appear to be derived from the same B-cell families, as evidenced by the comparison of the complementarity-determining regions of both the heavy and light chains versus germline.

UG1 optimization and purification

After the decision to focus on advancing UG1 over UG2-3 and because initial trial productions of UG1-3 scFv resulted in poor yields, we submitted the UG1 sequence to GENEART

for codon optimization for subsequent recombinant antibody expression in *E. coli*. The codon-optimized expression clone was designated gUG1. GENEART codon optimization resulted in high levels of expression of soluble secreted gUG1 in culture supernatant facilitating FPLC nickel-agarose purification of HIS-tagged gUG1 using a stepwise gradient elution with imidazole followed by desalting using FPLC gel permeation column. The fractions eluted from gel permeation were compared by silver stain to the post-nickel agarose sample and showed a high degree of purity with a band of approximately 25 kDa corresponding to chicken scFv (data not shown). Western blotting using anti-HA HRP confirmed the 25-kDa proteins as HA-tagged scFv (data not shown).

Flow cytometric characterization of gUG1-binding cells in peripheral blood

Purified, HA-tagged gUG1 was utilized in multicolor FACS to detect the frequency and surface-marker characteristics of gUG1-binding cells in PBMCs. A PE-labeled anti-HA

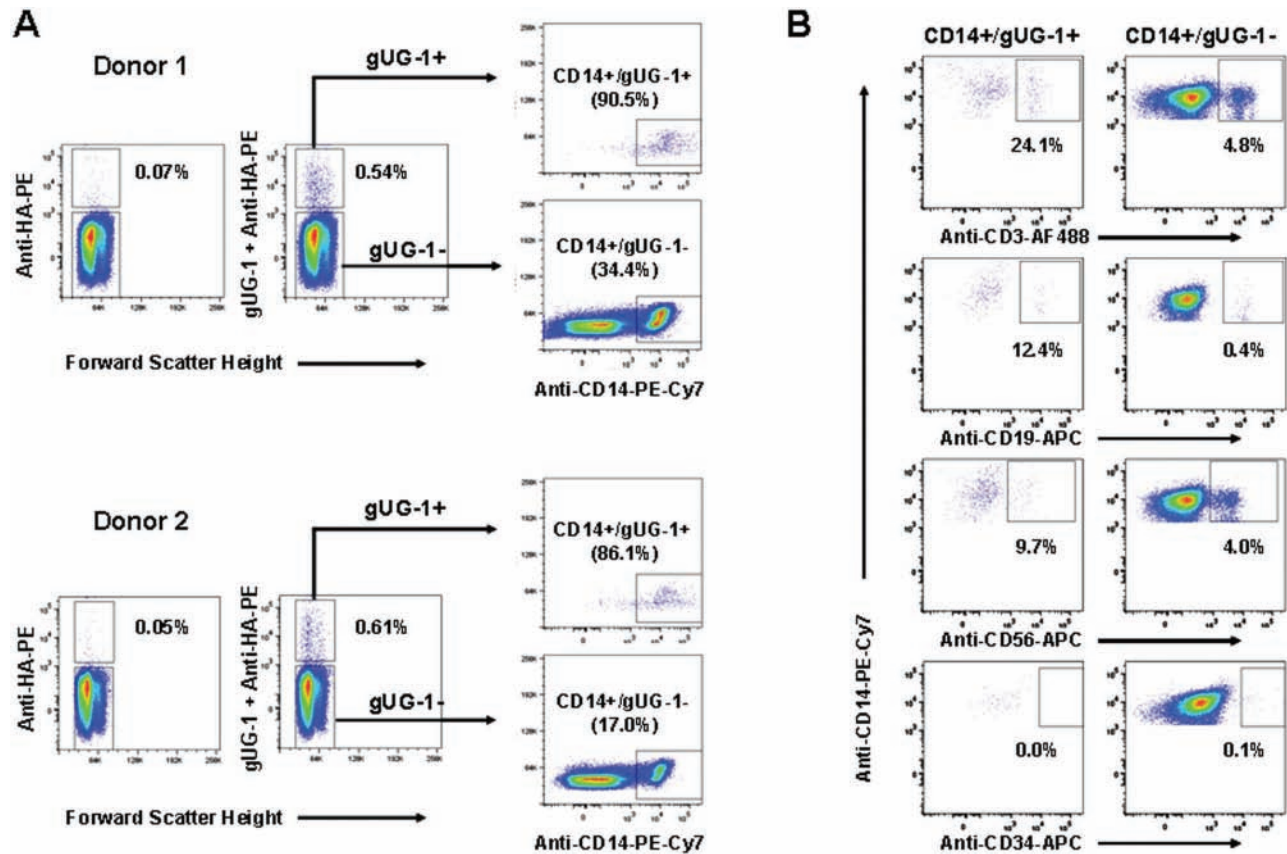


FIG. 5. Multicolor flow cytometric analysis of gUG-1-binding cells in peripheral blood. (a) Flow cytometry dot plots from four-color analyses of peripheral blood mononuclear cells (PBMCs) from two healthy adult donors are shown. Dot plots on the left illustrate staining of viable PBMCs for anti-HA-PE in the absence and presence of HA-tagged gUG-1 with proportions of anti-HA-PE+ cells indicated. Dot plots on the right illustrate staining for CD14 of the gUG-1+ and gUG-1- subpopulations with proportions of anti-CD14+ cells indicated. (b) Dot plots are shown of PBMCs from one of two healthy adult donors demonstrating CD14+/gUG-1+ and CD14+/gUG-1- cells co-stained for the T-cell marker CD3, the B-cell marker CD19, the NK-cell marker CD56, and the stem/progenitor cell marker CD34. Proportions that were positive for the relevant co-stain are indicated.

m0 monoclonal antibody was used as a secondary reagent to detect gUG1 cell surface binding with appropriate FcR blockade and dead cell exclusion. In preliminary titration experiments with PBMCs from two healthy adult volunteers, binding of gUG1 to a subset of PBMCs was observed with optimal staining compared with anti-HA-PE alone identified at 2.5 μ g of gUG1 per 5×10^5 cells (data not shown). Subsequently, gUG1 surface staining was combined, in four-color analyses, with staining for markers of major PBMC subsets to further analyze positively staining cells in two more healthy adult donors. As shown in **Figure 5a**, non-specific binding of anti-HA-PE in the absence of gUG1 was limited to <0.1% of viable PBMCs whereas the combination of gUG1 and anti-HA-PE resulted in staining of a distinct subpopulation of cells comprising between 0.5% and 0.6% of viable PBMCs. Co-staining for the monocyte-specific protein CD14 indicated that gUG1+ cells were highly enriched for this

marker in both samples being 91% and 86% CD14+ compared with 34% and 17% for the gUG1-negative cells. Combined co-staining for T-cell (CD3), B-cell (CD19), and NK-cell (CD56) markers indicated that gUG1+/CD14+ cells were more likely than gUG1-negative/CD14+ cells to co-express surface markers for these lymphocyte subtypes but did not express the stem/progenitor cell marker CD34. These results are shown for one donor in **Figure 5b**. Closely comparable results were also obtained for PBMCs from the second donor (data not shown).

A separate flow cytometric analysis of PBMCs was carried out to determine whether gUG1-binding cells preferentially expressed receptors for angiogenic factors (**Fig. 6**). As with prior PBMC samples, distinct populations of gUG1+/CD14+ and gUG1-/CD14+ cells were detectable. Co-staining with antibodies against the angiopoietin receptor Tie-2 (CD202b) and the VEGF receptor CD309 compared with isotype control antibody did

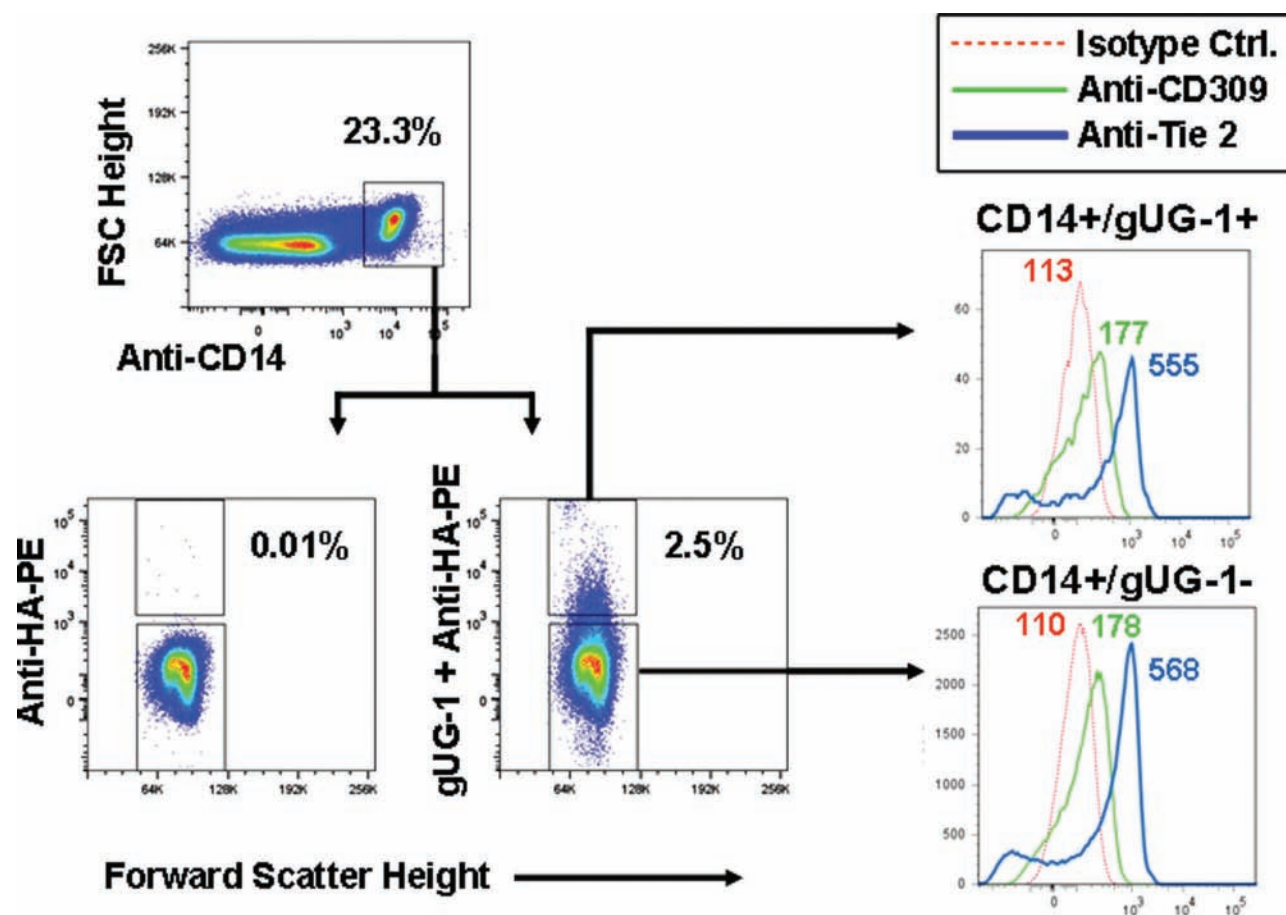


FIG. 6. Expression of proangiogenic receptors by gUG-1–positive and gUG-1–negative peripheral blood monocytes. Flow cytometry dot plots from four-color analyses of peripheral blood mononuclear cells (PBMCs) from one healthy adult donor are shown. CD14⁺ cells comprised 23.3% of the total viable PBMCs (upper left). Anti-HA staining of CD14⁺ cells in the absence and presence of gUG-1 are shown (lower and mid left) with the anti-HA⁺ proportions indicated. Histograms (right) demonstrate surface staining of CD14⁺/gUG-1⁺ and CD14⁺/gUG-1⁻ cells with antibodies against CD309 and Tie-2 compared with staining with isotype control antibody. Numerical values indicate the median fluorescence intensity of staining for the individual fluorochrome-labeled antibodies. Note that gUG-1⁺ cells are expressed as a proportion of CD14⁺ cells rather than of total viable PBMC as in **Figure 5**.

demonstrate expression of these receptors (Tie-2 > CD309) on gUG1⁺/CD14⁺ cells, but as shown in **Figure 6**, very similar levels of expression were also detectable on gUG1⁻/CD14⁺ cells. Overall, it was concluded from these analyses that gUG1 binds predominantly to a subset (approximately 2%–4%) of CD14⁺ monocytes that more frequently co-express lymphocyte markers compared with non-gUG1-binding monocytes but do not preferentially express receptors for proangiogenic factors.

DISCUSSION

We have successfully produced an immune chicken phage display library against live EPCs with angiogenic potential and identified three single-chain antibodies, named UG1-3. Cultured EPCs mainly consist of T lymphocytes and monocytes, as

shown here by us in single-color immunocytochemistry assays and as previously observed by others.^{14,15} These cells stimulated angiogenic tubuli formation of HUVECs in matrigel assays and expressed endothelial cell surface markers such as vWF and KDR. The immunization of chickens with intact cells provoked a distinct serum immunoglobulin response despite the lack of an adjuvant preparation. This might be explained by the following advantages of immune chicken phage display library technology: Avian evolution diverged from rodents and humans more than 200 million years ago, resulting in a great phylogenetic distance between birds and mammals.²³ It has been reported that the immunoglobulin response of chickens to highly conserved mammalian proteins is reliably robust, exhibits high affinity, and potentially targets a broad spectrum of epitopes on protein immunogens due to low amino acid homology between the

mammalian and avian proteins and due to the absence of some (mammalian) proteins in avians.^{17–21} The chicken immunoglobulin repertoire is ideally suited to antibody phage display, as chickens generate their immunoglobulin repertoire from a single set of V_H and V_L germline sequences,²⁵ which means that the entire antibody repertoire can be captured using only four PCR primers.^{17,25} This is in contrast with rodent immune hosts, which have diverse germline V-gene sequences and therefore require complex mixes of PCR primers.²⁶ In addition, the two V-gene germline sequences found in chickens are highly homologous to the human $V\lambda$ and V_H3 germline families, both of which are associated with creating V-domains that are highly stable, highly soluble, and display efficiently on phage.^{21,27,28} This leads to a more complete sampling of the encoded V-gene repertoire. Chicken scFvs can be readily produced in large quantities and are tagged for rapid isolation and detection using highly specific secondary antibodies. In addition, these scFvs can be converted to IgG^{29,30} and humanized.²⁷

Codon-optimized gUG1 had improved yields of recombinant protein that facilitated an additional FPLC gel permeation chromatography-based desalting step after nickel-agarose purification.

Using a fluorochrome-labeled antibody against HA-tagged recombinant gUG1, we successfully carried out a preliminary FACS analysis of the binding of this reagent to the surface of circulating leukocytes from healthy adult volunteers. Four-color analyses with rigorous exclusion of dead cells and non-specific artifacts allowed us to confidently determine co-expression of a gUG1 binding partner with a panel of basic markers for circulating monocytes, lymphocytes, and stem/progenitor cells. The results of flow cytometry of PBMCs from multiple donors clearly identify a low-frequency population of gUG1-binding cells that are almost exclusively CD14+. The target or targets for gUG-1 binding on these cells is unknown at present, and it should be acknowledged that, in the absence of a structurally matched, negative control single-chain construct, we cannot definitively prove that the staining patterns observed result from a specific binding of the combined heavy- and light-chain variable regions of gUG-1 to a single surface protein. Nonetheless, through rigorous exclusion of trivial, nonspecific staining patterns due to dead cells, doublets, autofluorescence, or secondary antibody binding, the results of this analysis confirm the selection of a single-chain chicken antibody with specificity for a distinct leukocyte subset that is in keeping with the monocytic origin of cultured EPCs.^{14,15} The gUG1+ subset was CD34-negative, arguing against a stem/progenitor phenotype, although further analysis, including cell sorting, will be necessary to examine their capacity for multilineage differentiation and self-renewal. Extended in vitro comparisons of gUG1+ and gUG1– cells will also be required to determine whether gUG1 binding identifies a monocytic subset with preferential or accelerated endothelial differentiation or with enhanced proangiogenic properties.

Although we hypothesized that gUG1+ cells may express higher levels of receptors for angiogenic peptides, this proved not to be the case for Tie-2 and CD309 expression when compared with other CD14+ leukocytes. Nonetheless, this does not rule out the presence of other angiogenic phenotype characteristics in the gUG1+ population, and future work will include a broader profile of the secreted products of purified gUG1+ cells and analyses of their potency in promoting new vessel formation in vitro and in vivo.

Finally, the higher proportion of gUG1+/CD14+ cells that co-expressed CD56, CD3, and CD19 is of interest. Although we cannot definitively exclude the possibility that the co-staining represented monocyte/lymphocyte doublets, back gating analysis indicated that this was very unlikely (not shown). Subsets of CD56+/CD14+ monocytes have been previously described,^{31,32} and it is possible that this observation represents a greater degree of functional compartmentalization or lineage plasticity among gUG1-binding monocytes.

Overall, this study illustrates the potential application of immune antibody phage display technology in the discovery of cell-specific antibodies to target cells including stem cells and progenitor cells. The advantages of the method described here are (a) the ability for rapid screening of the entire cell-surface marker repertoire in mixed populations of progenitor cells and stem cells; (b) the generation of antibodies against epitopes of surface markers on intact, live cells, thereby avoiding problems associated with screening libraries on antigens attached to plastic surfaces such as not correctly displayed structures of the target protein; and (c) the isolation of scFvs that may be used to pull down the target cell population of interest for enrichment and subsequent functional assays or to use these scFvs as targeting tools for nonviral gene delivery systems to endothelial progenitor cells. This protocol may be expanded toward screening of endothelial progenitor cells that commit to becoming late outgrowth cells, allowing for a detailed analysis of changes in the repertoire of cell-surface markers.

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