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Significant immunological obstacles are to be negated before xenotransplantation becomes a clinical reality. An initial rejection of transplanted vascularized xenograft is attributed to Galα1,3Galβ1,4GlcNAc-R (Galα1,3-Gal)–dependent and –independent mechanisms. Hitherto, no receptor molecule has been identified that could account for Galα1,3-Gal–independent rejection. In this study, we identify the tetraspanin CD82 as a receptor molecule for the Galα1,3-Gal–independent mechanism. We demonstrate that, in contrast to human undifferentiated myeloid cell lines, differentiated cell lines are capable of recognizing xenogeneic porcine aortic endothelial cells in a calcium-dependent manner. Transcriptome-wide analysis to identify the differentially expressed transcripts in these cells revealed that the most likely candidate of the Galα1,3-Gal–independent recognition moiety is the tetraspanin CD82. Abs to CD82 inhibited the calcium response and the subsequent activation invoked by xenogeneic encounter. Our data identify CD82 on innate immune cells as a major “xenogenicity sensor” and open new avenues of intervention to making xenotransplantation a clinical reality.

T

he acute shortage of donor organs leads to so many deaths of patients in dire need of transplantation. It is estimated that the global number of patients requiring heart transplantation is ∼800,000 whereas the total number of hearts transplanted in 2007 reached only 3,500 (1). One viable option for donor organ shortage is the use of animal organs as replacements, that is, xenotransplantation. Initially a transplanted organ between discordant species appears viable and healthy, but this is rapidly followed by hyperacute rejection attributed to xenoreactive natural Abs and complement activation (2, 3). Recipient xenoreactive natural Abs target Galα1,3-Gal, which “decorates” proteins and lipids of the transplanted organ endothelium (4, 5). These decorations are brought about by the enzyme α1,3-galactosyltransferase (GaIT), which is expressed in all mammals except humans, apes, and old world monkeys (6, 7). Many strategies have been employed to overcome hyperacute rejection. These include removal of the anti–Galα1,3-Gal Abs (8), accommodation (9), transgenesis (10, 11), and small interfering RNA silencing of the GalT (12). GaIT knockout (KO) donor organs gave a glimpse of hope through extending the life of the transplanted organ but succumbed to rejection, eventually albeit at a considerably later time (13, 14). Clinical xenotransplantation is controversial owing to the identified rejection problems and the possibility of xenozoonotic diseases (8, 15). Neutrophils and NK cells were identified as Galα1,3-Gal–independent players in xenograft rejection.

We and others have previously demonstrated the xenogeneic recognition and activation of neutrophils and NK cells by porcine aortic endothelial cells (POAECs) in the absence of xenoreactive natural Abs and complement activation through a calcium-dependent mechanism (16–19). The molecular mechanisms underlying such Galα1,3-Gal–independent recognition have yet to be determined. In this study, POAECs from wild-type (WT) and GaIT KO animals confirm that recognition of xenogeneic endothelial cells occurs independently of Galα1,3-Gal. Furthermore, we used three human myeloid cell lines (HL-60, THP-1, and KG-1) that, in their undifferentiated state, do not recognize xenogeneic endothelial cells as defined by the lack of calcium transients and reactive oxygen metabolite (ROM) production in response to POAECs GaIT KO and POAECs WT. However, when differentiated, these cells transiently raise their intracellular calcium and increase ROM production upon exposure to either POAECs GaIT KO or POAECs WT. To identify possible Galα1,3-Gal–independent sites mediating the recognition of xenogeneic endothelial cells, we used serial analysis of gene expression (SAGE). SAGE libraries of the myeloid cell lines were used to compare transcriptomics before and after differentiation with that in resting...
human naive neutrophils. This strategy yielded a number of transcripts that were 1) differentially expressed in all of the differentiated versus undifferentiated cell lines and 2) constitutively expressed in human naive neutrophils. Twelve differentially expressed transcripts were identified by this approach, with only six transcripts displaying consistent change in all three cell lines and in human naive neutrophils. Because the putative xenoreognition moieties should be both trans-plasma membrane proteins and associated with intracellular calcium release, only one of the six identified transcripts encoding the tetraspanin CD82 met the above criteria and therefore was considered the likely candidate mediating the Galα1,3Gal–independent recognition. This was confirmed by subsequent analysis that demonstrated that Abs to CD82 significantly inhibited both the calcium rise and ROM production in human naive neutrophils upon exposure to P0AECs GaIT KO and P0AECs WT. We therefore propose that a CD82-mediated interaction of innate immune cells with xenogeneic endothelial cells is one of the mechanisms employed to recognize interspecies xenogenic.

Materials and Methods

Materials

Fluo-3-acetoxymethylester (flu-3-AM) and fura-2-AM were purchased from Molecular Probes (Invitrogen, Carlsbad, CA). LightCycler DNA Master SYBR Green I was purchased from Roche Diagnostics (Mannheim, Germany). An I-SAGE/I-Long SAGE kit with magnetic stand, Platinum Taq DNA polymerase, and TRIzol solution were purchased from (Invitrogen). Cell lines were purchased from the American Type Culture Collection (Rockville, MD). Culture media (RPMI 1640 and DMEM) were purchased from Life Technologies (Grand Island, NY). Abs to von Willebrand factor were purchased from Sigma-Aldrich (St. Louis, MO), and acetylated low-density lipoprotein (Dif-Ac-LDL) was purchased from Biogenesis (Bournemouth U.K.). Abs to CD82 were purchased from Abcam (Cambridge, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). Anti–LFA-1α was purchased from R&D Systems (Minneapolis, MN). Anti–Galα1,3Gal Abs were prepared essentially as described previously (9). Secondary goat anti-mouse FITC-labeled Abs were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, and Alexa Fluor 647–labeled secondary Abs were purchased from Pierce. PMA, DMOS, dibutyryl cAMP, MTT, cell culture reagents, protease inhibitors, and other analytical-grade reagents were purchased from Sigma-Aldrich. Restriction enzymes NlaIII, MmlII, and Sph were purchased from New England Biolabs (Beverly, MA). Fluo-3-AM, Fura-2-AM, and luminol were dissolved in DMSO and delivered to the cells at a final concentration of 1, 1, and 1 μM, respectively, in a final DMSO concentration of 0.1%.

Preparation of endothelial cells

P0AECs (P304K-05) and human aortic endothelial cells (HAEcs: 304K-05a) were purchased from Cell Applications (San Diego, CA). HUVECs (CC-2517) were purchased from the Lonza Group (Basel, Switzerland). P0AECs and HAEcs were cultured and maintained in tissue culture medium from Cell Applications, whereas HUVECs were cultured and maintained in tissue culture medium purchased from Life Technologies. Cells were used from passages 2 through 10 in all experiments at a split ratio of 1:3. Additionally, arterial endothelial cells isolated from WT pigs and P0AECs derived from GaIT KO pigs were used when appropriate. These cells were provided by R. J. Hawley (formerly of Immune Biotherapeutics, Cambridge, MA). Cell lines were obtained and cultured as previously reported (26, 27). To test that endothelial cells were not activated during culture, IL-1 levels in conditioned medium were measured using ELISA (R&D Systems) and were consistently found to be negligible (<4 pg/ml).

Preparation of neutrophils

Human peripheral blood neutrophils were prepared by dextran sedimentation of heparinized whole blood obtained from healthy donors and centrifuged through Ficoll-Paque as described previously (16). Neutrophils were routinely tested for production of ROMs by luminol-dependent chemiluminescence (LDCL) for 10 min. Cells were considered naive and therefore suitable for experimentation only when no increase in LDCL was observed.

Preparation of cell Lines HL-60, KG-1, and THP-1

Acute promyelocytic leukemia HL-60 cell line (ATCC CCL-240), acute myelogenous leukemia KG-1 cell line (ATCC CCL-246), and acute monocytic leukemia THP-1 cell line (ATCC TIB-202) were purchased from the American Type Culture Collection. HL-60 and KG-1 cell lines were cultured in complete Iscove’s modified (American Type Culture Collection, catalog no. 30-2005) supplemented with 10% FBS (American Type Culture Collection, catalog no. 30-2020), penicillin (100 U/ml), and streptomycin (100 μg/ml). THP-1 cells were cultured in complete RPMI 1640 medium (American Type Culture Collection, catalog no. 30-2001) supplemented with 10% FBS (American Type Culture Collection, catalog no. 30-2020), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cell lines were maintained in a humidified incubator at 37°C with 5% CO2. HL-60 differentiation into neutrophil-like cells was performed by treatment of 2 × 106 cells/ml with 1.3% DMSO (Sigma-Aldrich, catalog no. D4540) in complete media for 6–8 d with media change every third day. Differentiation into neutrophil-like cells was ascertained by their ability to generate ROMs in response to stimulation by PMA (100 ng/ml) or the chemotactic peptide (500 μg/ml). This was detected by either the reduction of the soluble NBT to blue-black insoluble formazan and/or LDCL. For the former, 1 ml cell suspension was incubated for 20 min at 37°C with an equal volume of 0.2% NBT (Sigma-Aldrich) dissolved in PBS (pH 7.2; 0.15 M without Ca<sup>2+</sup>, Mg<sup>2+</sup>) in the presence of 200 ng of PMA. Differentiated cells contain formazan deposits as dark, irregularly shaped crystal inclusions in the cytoplasm. By day 6–9, 98% of the cells reduced NBT, and PMA stimulation and <5% of the cells were absent of PMA stimulation. THP-1 and KG-1 differentiation was performed as above but with treatment with dibutyl cAMP (500 μg/ml) and PMA (100 ng/ml) for 4 and 5 d, respectively (23). Differentiation was confirmed by ROM production as above.

Calcium measurements

Neutrophils or appropriate cell lines were loaded with fluo-3-AM (1 μM) as described previously (18). The cells were washed, placed on glass coverslips, and allowed to adhere for 15 min at room temperature. Coverslips were washed and then secured between two plates of a custom-designed coverslip holder, placed onto a heated microscope stage (37°C), and intracellular Ca<sup>2+</sup> concentration images were acquired at 1- to 2-s intervals. For each coverslip, 100 μl P0AECs WT and P0AECs GaIT KO, suspended in Krebs-HEPES buffer (pH 7.4) and containing 10<sup>6</sup> cells/ml, were added and image acquisition was continued for at least 5 min. Control experiments were carried out using equal numbers of HAEcs or HUVECs per coverslip. Images were analyzed using UltraVIEW confocal software (PerkinElmer, Cambridge, U.K.) and fluorescence intensity (from each cell) was transformed into absolute calcium levels as described previously (16). Because undifferentiated cell lines were nonadherent, calcium measurements were carried out using flowcytometric assays (PerkinElmer LS 55 luminescence spectrometer) with cells labeled with fura-2 AM as described previously (24). Experiments were analyzed using FL WinLab software (PerkinElmer). In experiments involving anti-CD82 Abs we used mouse monoclonal anti-human CD82 (TS22b, ab59509, lot GR 25852-1, Abcam).

Measurement of ROM production

The production of ROMs by neutrophils and the three cell lines were measured using LDCL on an FB12 single tube luminometer (Berdin Detection Systems/Tittertek Instruments, Huntsville, AL), essentially as described previously (18).

RNA isolation

Total cellular RNA was isolated from 1 to 2 × 10<sup>6</sup> differentiated/undifferentiated cell lines and human naive neutrophils using TRI Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s instructions. RNA integrity was routinely checked using 500 ng/ml RNA on 1% denaturing agarose gel.

Construction of 5′ Long SAGE libraries

SAGE was performed according to the SAGE detailed protocol version C and analyzed using SAGE analysis software version 4.5 (Johns Hopkins University, Baltimore, MD). In brief, 10 μg total RNA was bound to solid-phase oligo(dT) magnetic beads, and cDNA was synthesized directly on the oligo(dT) bead. Oligo(dT) bound to magnetic beads was used as a template for the first-strand cDNA synthesis, followed by the second-strand cDNA synthesis. The captured cDNAs were then digested with an “anchoring” restriction enzyme, NlaIII, which left a 3′ overhang. Complementary cDNA synthesis and NlaIII digestion was verified using PCR. The 3′ fragments were then isolated using magnetic beads, equally divided
into two pools and ligated to two different linkers, A or B. Both linkers contain the recognition sequence for a "tagging" restriction enzyme (type IIIs restriction enzyme), Mme. The tagging enzyme produced a staggered cut, offset by \( \sim 17 \) bp from the recognition sequence. The two linkers were ligated onto the NlaIII overhangs. The efficiency of ligation was assessed by PCR. Subsequent digestion with Mme released the adapter with a short tag of cDNA from the beads. These tags were then ligated tail-to-tail to form 130-bp ditags. The resulting ditags were PCR amplified using primers specific to each linker, pooled, precipitated, and gel purified. The linkers were released by digesting with NlaIII, and the resulting 34-bp ditags were gel purified, concatenated, and resolved on 8% (w/v) polyacrylamide gel. The high-molecular mass bands (300–500 bp, 500–800 bp, and 800–1 kb) were gel purified and cloned into SpHl-linearized pZfEO-1 vector (Invitrogen). Ligation products were transformed into One Shot TOP10 electrocompetent cells. Transformants were analyzed by colony PCR. Approximately 4000–5000 clones for each library were sequenced using M13 forward primer and analyzed on an Applied Biosystems DNA sequencing. Each concatemer insert results in a randomly organized “series” of ditags of \( \sim 34 \) bp, with each being flanked by the recognition sequence of the primary anchoring enzyme NlaIII CATG sequence that provides a “SAGE tag” specific to each expressed gene. Approximately 20–25 individual tags were produced per clone. SAGE software was used to convert these sequences into Long SAGE tags and tabulate tag abundances. Resultant SAGE tags were analyzed using the downloadable reference sequence database SAGEmap (25) from the National Center for Biotechnology Information Web site. By determining the frequency distribution of the total tag population, the statistical breakdown of the relative abundance of the different mRNAs expressed in the differentiated versus undifferentiated cell populations was obtained. Using this method we generated five libraries, including human naive neutrophils and differentiated and undifferentiated HL-60 and KG-1 cell lines (SAGE data were submitted to the Gene Expression Omnibus repository under accession no. GSE43211; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lzyffqkqokkgchc&acc=GSE43211). The differential expression results for THP-1 cells were from the Gene Expression Omnibus data repository at the National Center for Biotechnology Information (accession no. GSE1439). All SAGE tags identified for the three cell lines were aggregated for each gene across multiple tags. Differential expression significance for these aggregated counts was determined using a z test, where \( z = (x - \bar{x}) / \sigma \), and \( \sigma \) values were assigned as \( < 0.01 \) for \( z > 2.58 \) and as \( > 0.05 \) for \( z > 1.96 \), according to a univariate normal distribution. Subsequently, differentially expressed genes in all three cell lines (\( p < 0.05 \)) were considered.

Quantification of specific transcripts with LightCycler RT-PCR

RNA isolation. Total RNA was extracted from tissue culture cells using an RNaseasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The RNA quality was verified using formaldehyde gel. RNA concentrations were determined by absorption at 260-nm wavelength with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Real-time RT-PCR. Total RNA (2 \( \mu \)g) was reverse transcribed using a RT system from Promega (Madison, WI). The cDNA was then used as template for real-time PCR with gene-specific primers.

Gene-specific primers corresponding to the PCR targets were designed using Oligo 6 primer analysis (Applied Biosystems, Foster City, CA). The following oligonucleotides were selected: CD82 splice variant V1 (accession NM_002231, sense, 5'-GGTCTGTCATCTGTTGTTG-3'; antisense, 5'-CCAGAAAGCCCTACTTTCTC-3’); CD82 splice variant V2 (accession NM_001204844, sense, 5'-GGATGCCTGTGATGAGGC-3'; antisense, 5'-CCAGAAGGGCTGGACTTCT-3'); human GAPDH (accession NM_002046, sense, 5'-GTTGGAAGTTCGAGGAGC-3'; antisense, 5'-ATATCACTATAGGTCCAGCTT-3'); POAECs GAPDH (accession NM_L36535, sense, 5'-CTCGATGTTGAGGAACCT-3'; antisense, 5'-AGATGAGCTTTGGGCCCAC-3'); and POAECs GAPDH (accession NM_001206359, sense, 5'-GTCGACGTGGGTGACAT-3'; antisense, 5'-AGCTGGCAAGATGGTGTCC-3').

Conditions for real-time PCRs were first optimized using a gradient experiment. MgCl\(_2\) concentrations were reoptimized using the LightCycler protocol (Roche Molecular Biochemicals, Mannheim, Germany). Preliminary real-time RT-PCR experiments were performed with each primer pair to determine the annealing temperature that yielded the greatest amount of specific product with melting temperature separable from primer-dimer temperature. Standard curves were prepared for each run using known quantities of cDNA (10-fold dilutions beginning at 15 ng/\( \mu \)l) and primers for the gene of interest or GAPDH. LightCycler FastStart DNA Master SYBR Green I reaction mix containing 0.4 \( \mu \)M forward and reverse primers, 4 \( M \) MgCl\(_2\), and 1 \( \mu \)L LightCycler FastStart DNA Master SYBR Green I dye was used for quantitative real-time PCR analysis according to the manufacturer’s protocols (Roche Molecular Biochemicals). A volume of 1 \( \mu \)l from a 10-fold dilution of cDNA was added as the PCR template. A no-target control received 9 \( \mu \)l reaction mix and 1 \( \mu \)l water. PCR amplification was performed in triplicate wells. A four-step experimental run protocol was performed: 1) denaturation program (10 min at 95°C); 2) 45 cycles of four-segment amplifications (15 s at 95°C for 10 s, annealing (60-56°C) for 5 s, extension at 72°C for 10 s, and data acquisition at 83°C for 1 s (a temperature transition rate of 2°C/s with a single fluorescence measurement was used); 3) melting curve program (50–95°C, with heating rate of 0.1°C/s up to 98°C with continuous fluorescence measurement); and 4) a cooling program down to 40°C. Relative cycle number measurements were performed using standard curves for both target and GAPDH housekeeping gene. The second derivative maximum method was used for cycle threshold calculation from amplification curves. The respective concentration for any given sample was calculated using crossing-cycle analysis provided by the LightCycler software. For realistic quantifications, the starting amount of RNA was the same for all samples. Minor sampling errors were avoided by normalization against the housekeeping gene GAPDH.

Immunofluorescence and confocal microscopy

Detection of Galα1,3-Gal expression on POAECs was performed using baboon anti-Galα1,3-Gal Abs prepared from emulsified soluble Galα1,3-Gal with Hunter’s TiterMax adjuvant as described previously (9, 19). Briefly, POAECs WT or POAECs GalT KO (1 \( \times 10^6 \) cells/ml) seeded \( \sim 18 \) h previously on glass coverslips were fixed in 3.7% (v/v) formaldehyde (15 min at room temperature). Cells were washed thoroughly and Galα1,3-Gal structures were visualized by indirect immunofluorescence using baboon anti-Galα1,3-Gal IgG (1:250) and FITC-conjugated goat anti-human secondary Ab (1:500; Sigma-Aldrich). Cells were viewed by laser scanning confocal microscopy (Zeiss LSM 510 META software system; Zeiss, Jena, Germany). CD82 and LFA-1α double labeling was performed on live cells on ice. Briefly, 50 \( \mu \)l cell suspension (10 \( \times 10^6 \) cells/ml) was incubated with mouse monoclonal anti-human CD82 Ab (Abcam, lot 5561) and goat polyclonal anti-human LFA-1 (BD Biosciences, catalog no. AF1730) at 1:50 and 1:500 dilutions, respectively, for 1 h on ice. Cells were washed and treated with donkey anti-goat Alexa Fluor 647 secondary Ab (Molecular Probes, lot 93D1-1) for 30 min on ice at 1:500 dilution. The cells were washed thoroughly and then treated with goat anti-mouse FITC-labeled secondary Ab (Abcam, ab6785, lot 906955) at 1:500 dilution on ice for 30 min. The cells were then washed and spotted on the center of a coverslip, which was sandwiched between two plates of a specially designed holder, and viewed using a Zeiss LSM 510 META confocal microscope.

Flow cytometry

POAECs WT or POAECs GalT KO (1 \( \times 10^6 \) cells/ml) were labeled with anti-Galα1,3-Gal Abs (1:250) for 45 min on ice. The cells were washed three times (PBS [pH 7.2]) and then labeled with secondary Ab (FITC-conjugated goat anti-human IgG, 1:500; BD Biosciences, San Jose, CA, catalog no. 555988) for another 45 min on ice. Isotype IgG (IgG1/IgG3 isotype; BD Biosciences, catalog no. 349526) or secondary Ab alone was used as a control. Finally, the cells were washed with PBS, fixed in 1% paraformaldehyde, and analyzed by a BD FACSCalibur (BD Biosciences). Ten percent cells were analyzed in each sample to express the percentage of positive cells.

Sample preparation for Western blots

Appropriate cells (2 \( \times 10^6 \) cells/ml) were treated with 1.5 ml RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40 or 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 8.0], and protease inhibitors), scraped, transferred into a precooled centrifuge tube, and homogenized on ice. The homogenized cells were centrifuged at 16,000 \( \times g \) for 20 min. The supernatants were collected, washed with PBS, freshly made, and analyzed by Western blots. Samples were run in 4–12% SDS-PAGE gel, transferred onto nitrocellulose membranes, and washed thoroughly. Membranes were blocked (5% nonfat milk in TBST buffer) for 1 h and then probed with primary rabbit anti-human CD82 (H-173, Sc-5540; Santa Cruz Biotechnology) at a 1:200 dilution in blocking buffer overnight at 4°C. Membranes were washed twice with TBST and incubated in secondary anti-rabbit HRP-labeled Ab (W401B, 24947003; Promega) diluted 1:2500 with blocking buffer for 1 h at room temperature. Membranes were washed five times with TBST (10 min each) and probed by ECL (Amersham Pharmacia Biotech, Little Chalfont, U.K.).
Results
Calcium-dependent recognition of xenogeneic endothelial cells by human naive neutrophils is independent of Galα1,3-Gal

The absence of Galα1,3-Gal epitope in POAECS GalT KO was confirmed by confocal laser scanning microscopy and flow cytometry, which showed negative staining with baboon anti-Galα1,3-Gal-labeled POAECS GalT KO compared with POAECS WT (Fig. 1A). Further confirmation was obtained by the total absence of the enzyme GalT in POAECS GalT KO but not in POAECS WT (Fig. 1A). Exposure of human naive neutrophils to

FIGURE 1. (A) Fluorescence confocal micrographs of POAECS WT (upper top panel, left) and POAECS GalT KO (lower top panel, left) labeled with baboon anti–Galα1,3-Gal IgG prepared as described in Materials and Methods. The corresponding bright field micrographs (upper and lower top panel, center) and the superimposed images (upper and lower top panel, right). The middle panel shows flow cytometry data obtained from POAECS WT and POAECS GalT KO labeled with baboon anti–Galα1,3-Gal IgG, demonstrating Galα1,3-Gal–labeled POAECS WT (upper right quadrant) compared with lack of label in POAECS GalT KO cells (lower right quadrant). Corresponding control experiments with preimmune IgG fraction from baboon sera are shown on the upper and lower left quadrants, respectively. The bottom panel shows the expression of the GalT gene in POAECS WT but not in POAECS GalT KO cells using RT-PCR. Data are expressed as the GalT/GAPDH ratio. Experiments were performed as described in Materials and Methods. Data are representative of at least three independent experiments. (B) Calcium maps following exposure of human naive neutrophils to POAECS GalT KO (10^4/ml, top panel) at the indicated intervals. The bottom panel shows absolute intracellular calcium levels following xenogeneic exposure. The arrow indicates the time of addition of POAECS GalT KO. Data are expressed as means ± SEM (n = 53 cells). (C) Calcium maps in human naive neutrophils after exposure to POAECS WT (10^4/ml) (top left) and in the presence of saturating concentration of anti–Galα1,3-Gal IgG (top right). Calcium changes are color coded such that high calcium is indicated by warm colors. Bottom panels indicate the absolute calcium levels after exposure. Arrow indicates the times of addition of POAECS WT. Data are expressed as means ± SEM (n = 26 and 46 cells, respectively).
POAECs GalT KO (2 × 10^6/ml) resulted in a rise in their intracellular Ca^{2+} concentrations from the resting level of 69.5 ± 0.15 to 421 ± 20 nM before decaying back to pre–POAECs GalT KO encounter (Fig. 1A, 1B). This rise was largely dependent on release from intracellular stores, because parallel experiments performed in calcium-free medium in the presence of extracellular EGTA (1 mM) exhibited no significant difference in the extent of POAECs GalT KO–induced calcium rise. The calcium response was always asynchronous and heterogeneous. Similar results were obtained by POAECs WT where the neutrophil calcium rose to 499 ± 33 nM from the resting level of 70 ± 0.1 nM upon xenogeneic encounter (Fig. 1C). The calcium transient was unaffected by the presence of saturating concentrations of anti–Galα1,3-Gal Abs or the absence of xenoreactive natural Abs and complement. Moreover, neither HAECs nor HUVECs evoked any calcium rise in human naive neutrophils (16).

**Activation of human naive neutrophils by POAECs GalT KO and POAECs WT but not by allogeneic endothelial cells**

Activation of human naive neutrophils following xenogeneic encounter was tested by measuring ROM production using LDCL. In a series of experiments we found that POAECs GalT KO and POAECs WT elicited ROM production in human naive neutrophils (Fig. 2A). In contrast, neither HAECs nor HUVECs exhibited any effects on ROM production (Fig. 2A). Parallel experiments in the presence of saturating concentrations of Abs to Galα1,3-Gal Abs or the absence of xenoreactive natural Abs and complement. Additionally, these undifferentiated cells failed to produce ROMs upon exposure to the xenogeneic endothelial cells. In contrast, differentiated HL-60 cells displayed a transient calcium rise, with levels reaching 274 ± 3 nM from a resting level of 70 ± 0.01 nM after exposure to POAECs WT (Fig. 3A), and they were concomitant with ROM production (Supplemental Fig. 3). This suggested that differentiation was a prerequisite for the xenorecognition. This was further confirmed by the results obtained with THP-1 cells, which upon treatment with dibutyryl cAMP differentiate into neutrophil-like cells (Fig. 3B), and KG-1 cells, which upon treatment with PMA differentiate into monocyte-like cells (Fig. 3C).

**Undifferentiated human cell lines HL-60, THP-1, and KG-1 do not recognize xenogeneic endothelium unless differentiated into neutrophil-like or monocyte-like cells**

The question arose as to the identity of the Galα1,3-Gal–independent sites mediating the recognition of xenogeneic endothelial cells by human naive neutrophils. Because both POAECs GalT KO and POAECs WT xenogeneic endothelial cells were recognized by human naive neutrophils, and because the latter are terminally differentiated and therefore should possess all of the necessary components for such recognition, we investigated the ability of undifferentiated human myeloid cell lines to recognize xenogeneic endothelial cells. We tested three cell lines: HL-60 and THP-1 cells, which differentiate into neutrophil-like cells, and KG-1 cells, which differentiate into monocyte-like cells (26–28).

**Identification of the Galα1,3-Gal–independent xenogeneic recognition moieties by transcriptomics**

Because only differentiated cell lines and human naive neutrophils were able to recognize and respond to encounters with xenogeneic POAECs WT, the possibility existed that common molecular moieties in the four cell types may be responsible for this recognition. We therefore used SAGE to identify differentially expressed transcripts in the three cell lines and in human naive neutrophils. We used a snapshot approach of mRNA transcripts of undifferentiated and differentiated HL-60 and KG-1 cells, within which our recognition moieties were expected to exist. Undifferentiated HL-60 cells exhibited 14,578 transcripts after sequencing 20,261 tags, whereas differentiated HL-60 cells exhibited 16,277 transcripts following 18,206 sequenced tags. Of those transcripts, 248 were significantly differentially expressed (p ≤ 0.05) (Fig. 4). Similarly, undifferentiated KG-1 cells exhibited 31,311 transcripts obtained from 38,793 sequenced tags compared with 22,084 transcripts obtained from 29,810 sequenced tags in the differentiated KG-1 cells. Of all transcripts in KG-1 cells (undifferentiated and differentiated), 651 were significantly differentially expressed (Fig. 4). The differentially expressed transcripts from both cell types were then compared with differentially expressed transcripts of the THP-1 cell line available from the Gene Expression Omnibus data repository at the National Center for Biotechnology Information (accession no. GSE1439). This approach identified 12 differentially and significantly expressed transcripts (p ≤ 0.05) common to all three cell lines as they differentiate into neutrophil-/monocyte-like cells. Six transcripts displayed levels of expression that were not consistent in all three cell lines and were therefore excluded (Fig. 4, Table I), leaving six differentially expressed transcripts that were consistently upregulated in the three cell lines and were also expressed in the human naive neutrophils. Because our targets were expected to be associated with the plasma membrane, we used the Database for Annotation, Visualization and Integrated Discovery version 6.7 (http://david.abcc.ncifcrf.gov/), GOstat software and goa_hu-
man database (http://gostat.wehi.edu.au/), Ingenuity Pathway Analysis (https://analysis.ingenuity.com/pa/public/security.jsp), and Pathway Studio (http://www.ariadnegenomics.com/support/pathway-studio-8/) to analyze the cellular locations of the six transcripts identified above. Five of these transcripts, namely ferritin L chain, ferritin H chain, γ-actin, creatine kinase, and adenylate cyclase–associated protein-1, were all assigned a cytoplasmic location and were therefore excluded. This left the tetraspanin CD82 as the only differentially expressed trans-plasma membrane protein that is associated with the ability of differentiated cell lines and human naive neutrophils to recognize xenogeneic endothelial cells independently of Galα1,3-Gal (Fig. 4).

**Confirmation of SAGE results at the mRNA and protein levels**

To confirm the differential expression of CD82 transcripts we used quantitative RT-PCR and Western blot analysis on samples from undifferentiated and differentiated cell lines and human naive neutrophils. We found that in the undifferentiated HL-60 cells, the ratio of CD82 mRNA transcript relative to GAPDH increased from 2.40 ± 0.03 to 20.74 ± 0.13 upon differentiation. Similar results were obtained with the other two cell lines, KG-1 and THP-1 (Fig. 5A). These changes in the message levels were echoed by respective increased protein levels in Western blot experiments (Fig. 5A). Localization of the expressed CD82 was examined by confocal microscopy of live human naive neutrophils and found to be associated with the plasma membrane (Fig. 5B). Further confirmation of this location was provided by colocalization of CD82 with the adhesion molecule LFA-1α in dual-labeled live neutrophil experiments (Fig. 5C).

**Inhibition of xenogeneic recognition by anti-CD82 Abs**

Because SAGE, quantitative RT-PCR, Western blots, and confocal data identified CD82 as the likely candidate for xenogeneic recognition, we argued that blocking CD82 should inhibit recognition of POAECs WT by human naive neutrophils. In a series of experiments neutrophils were exposed to anti-CD82 Abs (1 μg/ml for 15 min at room temperature) prior to xenogeneic contact. This treatment significantly reduced POAECs WT–induced calcium from 482 ± 24 to 183 ± 12 nM (p ≤ 0.0001, Fig. 6A–C, Supplemental Figs. 1, 2) with a concomitant decrease in ROM production (Fig. 6D).
endothelial cells by human naive neutrophils occurs in a Gal–dependent recognition/activation mechanism, we used myeloid cell lines expressing relatively low levels of CD82 to evoke a calcium transient or ROM production in response to POAECs WT or POAECs GalT KO; 2) differentiated myeloid cell lines and human naive neutrophils expressing relatively higher levels of CD82 respond to xenogeneic POAECs WT and POAECs GalT KO; and 3) Abs to CD82 inhibit both calcium transients and ROM production in response to xenogeneic contact.

CD82, also known as C33 Ag or KAI1 and originally identified as a marker for activation/differentiation of mononuclear cells (31), is a member of the tetraspanin family of proteins responsible for divergent cellular activities, including activation (32), differentiation (33), motility (34), adhesion (35), signaling (36), fusion (37), and metastasis (38). They are highly conserved and can be found in species as disparate as fungi and mammals (39). In humans, CD82 is expressed in many cell types, including lymphocytes (40), granulocytes (41), epithelial cells (42), platelets (43), endothelial cells (44), and fibroblasts (45). Thirty-four mammalian tetraspanins were identified, with 33 expressed in humans (39). All have four transmembrane domains with cytosolic N- and C-terminal regions and two extracellular domains with a conserved CCG motif (39). CD82 exists as two isoforms resulting from two terminal regions and two extracellular domains with a conserved CCG motif (39). CD82 is expressed in many cell types, including lymphocytes (40), granulocytes (41), epithelial cells (42), platelets (43), endothelial cells (44), and fibroblasts (45). Thirty-four mammalian tetraspanins were identified, with 33 expressed in humans (39).

Discussion

We and others have previously reported the activation of human naive neutrophils by POAECs in the absence of xenoreactive natural Abs and complement and under conditions where the Galα1,3-Gal binding sites were blocked (16–19). In the present study we demonstrate that human naive neutrophils are activated not only by xenogeneic POAECs WT but also by POAECs GalT KO. Allogeneic HAECs or HUVECs, alternatively, do not invoke activation. This suggests that the recognition of xenogeneic endothelial cells by human naive neutrophils occurs in a Galα1,3-Gal–independent fashion. The presence of anti–non-Gal xenoreactive Abs in humans and nonhuman primates suggests the involvement of non-Gal recognition sites in the rejection process. Non-Gal Ags have been implicated in acute humoral xenograft rejection (29). Moreover, Saethre et al. (30) identified anti-Hanganutziu–Deicher Abs and others targeting the non-Gal binding sites on the xenogeneic endothelial cells isolated from POAECs GalT KO animals. The question, however, remains as to whether these non-Gal epitopes are involved in the xenorecognition and activation of human naive neutrophils by POAECs.

To gain insight into the molecular moieties involved in this non-Gal–dependent recognition/activation mechanism, we used myeloid cell lines that in their undifferentiated state are not activated by xenogeneic endothelial cells and only become activated after differentiation into neutrophil-like or monocyte-like cells. SAGE analysis of these cells and of resting human naive neutrophils revealed six transcripts that were consistently expressed in the differentiated cell lines and thus are the likely candidates responsible for xenorecognition and subsequent activation of these cell lines. Of these six transcripts, CD82 was identified as the likely candidate responsible for xenoendothelial cell recognition. Three lines of evidence support such a claim: 1) undifferentiated myeloid cell lines expressing relatively low levels of CD82 do not evoke a calcium transient or ROM production in response to POAECs WT or POAECs GalT KO; 2) differentiated myeloid cell lines and human naive neutrophils expressing relatively higher levels of CD82 respond to xenogeneic POAECs WT and POAECs GalT KO; and 3) Abs to CD82 inhibit both calcium transients and ROM production in response to xenogeneic contact.

Table I. Transcriptome analysis using SAGE libraries

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>SAGE Tags</th>
<th>HL-60</th>
<th>KG-1</th>
<th>THP-1</th>
<th>Neutrophil Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD82</td>
<td>CD82 molecule</td>
<td>GTGAAACCCCGTCTCTA</td>
<td>1.63</td>
<td>1.55</td>
<td>1.50</td>
<td>58.81</td>
</tr>
<tr>
<td>FTL</td>
<td>Ferritin, light polypeptide</td>
<td>CCCCCCGTCTCTCA</td>
<td>3.07</td>
<td>1.59</td>
<td>1.65</td>
<td>255.07</td>
</tr>
<tr>
<td>CKB</td>
<td>Creatine kinase, brain</td>
<td>GTGACACCACCGGGC</td>
<td>1.55</td>
<td>1.49</td>
<td>1.73</td>
<td>83.68</td>
</tr>
<tr>
<td>FTH1</td>
<td>Ferritin, heavy polypeptide 1</td>
<td>TTTGGGTCTCTTCCTACC</td>
<td>2.59</td>
<td>3.95</td>
<td>1.62</td>
<td>517.23</td>
</tr>
<tr>
<td>ACTG1</td>
<td>Actin, γ1</td>
<td>CTAGCTCTCAGAAGACTG</td>
<td>1.70</td>
<td>1.43</td>
<td>1.40</td>
<td>38.48</td>
</tr>
<tr>
<td>CAP1</td>
<td>Adenylate cyclase–associated protein 1</td>
<td>TCTACGTACCTGATTGCTT</td>
<td>1.96</td>
<td>2.16</td>
<td>1.82</td>
<td>77.11</td>
</tr>
<tr>
<td>TPT1</td>
<td>Tumor protein, translationally controlled 1</td>
<td>TAGTGGTCTTCAAATAATGA</td>
<td>1.59</td>
<td>2.52</td>
<td>1.50</td>
<td>49.53</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH</td>
<td>TACACGTGAATAAATAC</td>
<td>1.41 – 1.46</td>
<td>2.10</td>
<td>1.50</td>
<td>42.99</td>
</tr>
<tr>
<td>RPS20</td>
<td>Ribosomal protein S20</td>
<td>CGGCTTAGAAGGCG</td>
<td>–1.87 – 1.20</td>
<td>1.40</td>
<td>33.64</td>
<td></td>
</tr>
<tr>
<td>RPL38</td>
<td>Ribosomal protein L38</td>
<td>GCCAGGAGGCGCCTG</td>
<td>–1.90 – 1.42</td>
<td>1.64</td>
<td>30.00</td>
<td></td>
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<tr>
<td>RPS3A</td>
<td>Ribosomal protein S3A</td>
<td>GTGAGGAGGAGGCTCTT</td>
<td>–1.52 – 2.24</td>
<td>1.50</td>
<td>31.72</td>
<td></td>
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<tr>
<td>RPL34</td>
<td>Ribosomal protein L34</td>
<td>TGGCTCTAATGTGCTTC</td>
<td>–1.81 – 2.06</td>
<td>1.50</td>
<td>17.00</td>
<td></td>
</tr>
</tbody>
</table>

The analysis revealed 12 differentially expressed transcripts. Only six transcripts exhibited a consistent upward trend in the three cell lines upon differentiation and are present in the human naive neutrophils. Positive change is noted by boldface and negative change is noted by italics.
Whether such immune synapse occurs as part of the xenogeneic recognition mechanism is yet to be determined. It is tempting to speculate that the organizing capability of tetraspanins may direct the assembly of an innate immune synapse at the xenogeneic recognition milieu and the corresponding binding site on the membrane of the xenogeneic endothelial cell, thus initiating intercellular crosstalk through mobilization of intracellular calcium in both cell types. Intracellular calcium transients have been demonstrated in POAECs in response to xenogeneic encounter with human naive neutrophils and NK cells (16, 19). Our present data suggest a role for tetraspanins as xenogeneic sensors analogous to the role of TCR/MHC class II in allogeneic recognition.

Calcium involvement in tetraspanin signaling has been demonstrated through the use of Abs to the various tetraspanins. In platelets, mAbs to CD9 induced calcium transients through Fcγ II receptors (56). In contrast in human monocytes and B cells, calcium signaling was thought to occur via direct crosslinking of F(ab′)2 fragments of anti-CD53 Abs (57). Lebel-Binay et al. (31) have demonstrated that anti-CD82 Abs evoked calcium changes in the monocytic cell line U937 through dual binding to CD82 and Fcγ receptors. In our study, pretreatment of neutrophils with anti-CD82 Abs had no apparent effect on resting calcium homeostasis, whereas POAEC-induced calcium rises were significantly reduced. This apparent discrepancy may be due to different source of Abs and different...
cell types. Interestingly, human naive neutrophils lost their adhesive capabilities following anti-CD82 Ab treatment (data not shown).

The question as to the CD82 counterpart on POAEcs is yet to be determined. A clue may be provided by the presence of anti–non-Gal xenoreactive Abs in human and nonhuman primates. It is estimated that 10% of total anti-porcine Abs are targeted against the non-Gal moieties (30). Interestingly, GalT KO kidneys have been demonstrated to elicit xenointobody response in nonhuman primates (29, 58). Although no clear targets were identified, carbohydrate Ags on the xenogeneic endothelium might be a plausible one. The presence of anti-Hanganutziu–Deicher xenobodies suggests a role for sialic acid Ags. Sialic acid normally exists as N-acetylenuronic acid or N-glucolylneuramic acid. The latter is not expressed in humans (30). The association of CD82 and other tetraspans with gangliosides is known to modulate cellular signaling (59, 60). Whether CD82 on human neutrophils interacts directly with the sialic acid–containing structures on the porcine endothelium remains to be answered. Additionally, the significance of this “newly identified receptor” to the overall rejection process must be considered, especially in the presence of anti-porcine Abs, complement, and platelets. Tetraspans are membrane organizers and are associated with a variety of molecules, including complement receptor 2 (51), glycosynaps (59), and gangliosides, which are avid organizers of CD82-enriched microdomains (60).

Although the data presented in this study assign a new role for CD82 in xenoreognition and may therefore suggest that its downregulation is desirable for xenograft survival, such downregulation has been associated with increased metastasis in a number of human cancers, including colorectal cancers (61), squamous cell carcinoma (62), prostate cancer (38), breast cancer (63), and hepatocellular carcinoma (64, 65). The question as to how CD82, a tumor suppressor protein, mediates xenoreognition is yet to be addressed. Tumor metastasis suppression by CD82 is thought to occur by stabilizing E-cadherin/β-catenin complex formation (66), upregulation of Sprouty2 (67), maturation of xenogeneic endothelial cells and human naïve natural killer cells.

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Disclosures

The authors have no financial conflicts of interest.

References


