Genomic/proteomic analyses of dexamethasone-treated human trabecular meshwork cells reveal a role for GULP1 and ABR in phagocytosis

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Purpose: The purpose of this study is to examine the expression profile of genes related to integrin-mediated phagocytosis that are altered by dexamethasone (DEX) and/or αvβ3 integrin signaling to gain a better understanding of the molecular basis of phagocytosis and the pathophysiology of glucocorticoid-induced ocular hypertension.

Methods: RNA and cell lysates were obtained from human trabecular meshwork (HTM) cells incubated with and without DEX for 4–5 d. The relative level of gene expression was evaluated using the Affymetrix Gene Chip® human gene microarray and quantitative PCR (qPCR). Changes in protein expression were validated using western blots or FACS analyses. The involvement of proteins in phagocytosis was determined using siRNA to knock down the expression of these proteins in an immortalized TM-1 cell line. Changes in the phagocytic activity were measured using pHrodo™-labeled S. aureus bioparticles followed by immunofluorescence microscopy. The effect of αvβ3 integrin expression and activity on GULP1 mRNA levels was measured using qPCR in TM-1 cells overexpressing wild type or constitutively active αvβ3 integrin.

Results: Gene microarrays revealed statistically significant differences (>2 fold) in the expression of seven genes known to be involved in phagocytosis. Three genes (CD36, ABR, and GULP1) were downregulated, while four genes (ITGB3, CHN1, PIK3R1, and MFGES8) were upregulated. The genes were either associated with modulating RAC1 activity (ABR and CHN1) or integrin signaling (CD36, GULP1, ITGB3, PIK3R1, and MFGES8). Another gene, SIRPA, was also downregulated (1.6 fold) but only in one cell strain. qPCR and western blot analyses verified that DEX caused a decrease in SIRPA and GULP1 mRNA and their protein levels, while levels of CHN1 mRNA and its protein were upregulated by DEX. qPCR showed that although ABR mRNA was downregulated compared to non-treated controls after 5 d of treatment with DEX, no change at the protein level was detected. qPCR analysis also revealed that DEX caused an increase in MFGES8 mRNA levels. The levels of CD36 mRNA and protein varied between cell strains treated with DEX and were not statistically different compared to controls. The knockdown of GULP1 and ABR using siRNAs decreased phagocytosis by 40%. Interestingly, GULP1 mRNA levels were also decreased by 60% when αvβ3 integrin was overexpressed in TM-1 cells.

Conclusion: The DEX-induced inhibition of phagocytosis may be caused by the downregulation of ABR and GULP1 disrupting the αvβ3 integrin/RAC1-mediated engulfment pathway. The downregulation of GULP1 by αvβ3 integrin further suggests that this integrin may be a negative regulator of phagocytosis by transcriptionally downregulating proteins needed for phagocytosis. In summary, these results represent new insights into the effects of glucocorticoids and integrin signaling on the phagocytic process in the TM.

The phagocytic properties of trabecular meshwork (TM) cells are thought to play an important role in maintaining intraocular pressure by keeping the outflow pathway free of cellular debris and degraded extracellular matrix proteins that can restrict outflow and cause an elevation in intraocular pressure [1,2]. Abnormalities in the phagocytic properties of TM cells are believed to contribute to several different glaucomas, including exfoliation, pigmentary, and steroid-induced glaucoma [3,4]. Despite its importance, we know very little about the molecular components that control phagocytosis in TM cells.

Phagocytosis is a complex, highly orchestrated process that is divided into several steps and involves multiple intracellular and extracellular components [5,6]. Extracellular soluble factors called eat-me signals help identify the target to be engulfed; these are usually ligands for the engulfment receptors on phagocytes. They act as bridging molecules that mediate the phagocytic process between the phagocyte and its target. Once engulfment receptors on the phagocyte bind the debris either directly or indirectly via the soluble eat-me...
molecules, the engulfment process is triggered. The engagement of the engulfment receptors also activates signaling pathways that trigger the rearrangement of cytoskeletal elements responsible for the formation of the phagocytic cup. In most cases, these signaling pathways involve the small GTPase called RAC1 [7] that activates the phagocytic process and the GTPase RHOG that turns it off [8-10]. Not all the engulfment receptors are expressed on every phagocyte, and tissue-specific differences are observed. Nevertheless, it is generally accepted that multiple modes of recognition and coordinated actions of engulfment receptors and signaling complexes are involved to contend with the various physiologic circumstances a cell confronts.

To date, the signaling pathways that mediate the phagocytic process in TM cells appear to involve pathways commonly found in other phagocytic cell types, such as macrophages or retinal pigment epithelial (RPE) cells [9]. Recent studies show that phagocytosis in TM cells is a RAC1-mediated process that utilizes an αvβ5 integrin/FAK signaling pathway [11,12] similar to that observed in RPE cells [13]. The downstream modulators of αvβ5 integrin-mediated signaling that regulate RAC1 activity during phagocytosis involve the guanine nucleotide exchange factor (GEF) TIAM1 and the ELMO2/ILK complex that activates RHOG [12]. This phagocytic process is inhibited when the αvβ3 integrin is upregulated and activated and following treatment with the glucocorticoid dexamethasone (DEX) [11]. However, how αvβ3 integrin signaling and/or DEX treatment inhibits this process is still unknown.

Here, we investigated how DEX and the DEX-induced overexpression of αvβ3 integrin could inhibit the components involved in phagocytosis in TM cells downstream of αvβ5 integrin/FAK signaling. To understand the molecular mechanism(s) involved, we compared proteins affected by DEX with those in cells overexpressing αvβ3 integrin. We demonstrated that DEX did not affect to detect the expression levels of proteins known to be involved in αvβ5 integrin-mediated phagocytosis. Rather, it altered the expression of several genes known to mediate the activity of RAC1 (CHN1 and ABR) and integrin signaling (ITGB3, GULPI, PIK3R1, CD36, and MPGE8). Of those affected, only GULPI mRNA expression was altered in both DEX-treated HTM cells and cells overexpressing αvβ3 integrin, suggesting that this may be the common link between phagocytosis inhibited by either DEX or αvβ3 integrin signaling. Understanding how DEX and αvβ3 integrins inhibit phagocytosis in TM cells should provide insight into novel approaches and therapies to manage the signaling pathways governing this normal TM function.

METHODS

Materials: The monoclonal antibodies for β-actin (ab8227), SIRP-α (ab191419), α-chimaerin (ab156869), CD47 (ab3283), and CD36 (ab23680) were purchased from Abcam (Cambridge, MA). The αvβ3 integrin antibody (LM609; mAb1976) and FLAG tag antibody (M2; F1804) were purchased from Millipore Sigma (Burlington, MA). A monoclonal antibody for GULPI/CED-6 (NB100–1042) was purchased from Novus Biologicals (Littleton, CO). IRDye800-conjugated secondary antibodies (rabbit and mouse) were purchased from Li-Cor Biosciences (Lincoln, NE), pHrodo™ Red S. aureus bioparticles, Hoescht 33342 nuclear stain, and Cell-Mask Green were purchased from Invitrogen Life Technologies (Carlsbad, CA). siRNAs against human ITGB5, GULPI/CED6, ABR, and SIRP4 and a non-targeting siRNA were purchased from Dharmacon (Lafayette, CO). The pcDNA3.1 plasmid expressing FLAG-tagged (DYKDDDK) CHN1 was purchased from GenScript (Ohu26717D; Piscataway, NJ) and validated by DNA sequencing. The forward and reverse primers used for the sequencing were 5’-TAA TAC GAC TCA CTA TAG GG-3’ and 5’-CCT CGA CTG TGC CTT CTA-3’, respectively.

Cell culture: Strains of normal human trabecular meshwork (HTM) cells were isolated from two 27-year-old female donors and a 25-year-old male donor as previously described [14-16]. The N27TM-2, N27TM-4, and N27TM-5 strains were isolated from one 27-year-old donor, and the N27TM-6 cell strain was isolated from the other 27-year-old donor. The N25TM-8 and N25TM-10 cell strains were isolated from the 25-year-old donor. Cells were validated as previously described using morphology, glucocorticoid responsiveness, and upregulation of myocilin by DEX as criteria [14,17,18]. Cells were grown to confluency in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO) containing 15% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (Sigma), 1% amphotericin B (Mediatech, Herndon, VA), 0.05% gentamicin (Mediatech), and 1 ng/ml FGF-2 (Peprotech, Rocky Hill, NJ). After reaching confluency, HTM monolayers were cultured in the presence of 500nM DEX dissolved in 0.1% ethanol or vehicle control (0.1% ethanol) for 4–5 d in low-glucose media containing 10% FBS, 2 mM L-glutamine, 1% amphotericin B, and 0.05% gentamicin.

Imortalized human TM-1 cell lines expressing wild type (WT) or constitutively active (CA) αvβ3 integrin were established as previously described [11,17]. Cells were grown in low-glucose DMEM containing 2 mM L-glutamine, 1% amphotericin B, 0.05% gentamicin, and 10% FBS. Puromycin (2 ug/ml; DOT Scientific, Inc., Burton MI) was added to keep
selective pressure on these cell lines. Cell strains and lines were validated using STR analyses (see Appendix 1).

Microarray analysis: Microarray data generated in a previous study were re-analyzed for changes in genes involved in phagocytosis [19]. The HTM cells (N27TM-2 and N27TM-4) were dosed daily 24 h after confluency with either 500 nM DEX or 0.1% EtOH (vehicle) for 7 d. Changes in gene expression in the N27TM-2 and N27TM-4 strains were independently analyzed five and three times, respectively. Poly-A RNA controls were prepared as previously described using the Affymetrix® GeneChip® Eukaryotic Poly-A RNA Control Kit [19]. The integrity of the RNA used in the gene microarray was checked using an Agilent 2100 BioAnalyzer and was found to be intact. Appendix 2 in the supplement shows representative electropherograms of the RNA integrity. The RNA integrity number (RIN) scores for the RNA were between 8 and 10 for both the DEX- and EtOH-treated samples, and the 260/280 ratio was 2.04. cDNA synthesis was initiated using the GeneChip® WT cDNA Synthesis Kit as previously described [19]. The single-stranded cDNA was then fragmented and labeled using the GeneChip® WT Terminal Labeling Kit. The labeled, single-stranded cDNA samples were taken to the University of Wisconsin Biotechnology Center for hybridization, washing, and staining. The analyses were performed by the University of Wisconsin Biotechnology Center using an Affymetrix GeneChip Human Gene 1.0 ST Array containing 764,885 distinct probes representing 28,869 genes (November 2006 version). Statistical analysis of the raw data was done using Bioconductor in R, which uses moderated t-statistics [20] to generate the list of genes with a p value ≤0.05. DAVID Bioinformatics Resources was used to identify gene names from the probe IDs on the array. The results were compared to those of a previous published study [21] that used the U133A Affymetrix Gene Chip (June 2005 version). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE124114.

Western blot analysis: HTM cells (N25TM-8) were lysed for 10 min at 4 °C with a RIPA lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% NP-40, 0.25% deoxycholate) containing a Halt phosphatase inhibitor cocktail and a Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Cell debris was removed by centrifugation at 10,000 × g, and the protein concentration was determined using a bicinechonic acid assay (Thermo Scientific). Whole-cell lysates from DEX-treated and EtOH-treated cells were separated on 10% SDS–PAGE gels and transferred to an Immobilon-FL membrane (Millipore, Billerica, MA). Membranes were blocked with either 3% BSA in TBS/0.05% Tween-20 (TBST) for β-actin staining or with 5% milk in TBST for SIRPA, α-chimaerin, and GULP1 staining. Membranes were incubated overnight with a 1:1000 dilution of the primary antibody in TBST containing either 3% BSA or 5% milk. Membranes were then washed with TBST and incubated for 1 h with an anti-mouse or anti-goat IRDye® 800CW-conjugated secondary Ab in TBST containing 1% BSA and 0.01% SDS. Labeled membranes were viewed using the Li-Cor Odyssey imaging system. Each blot was replicated three times using cell lysates independently obtained from three different experiments.

RNA isolation, reverse transcription, and quantitative PCR: RNA from confluent cultures of five different HTM cell strains grown as described above and treated with 500 nM DEX or 0.1% EtOH was isolated using the QIAshredder and RNeasy Plus Mini Kits (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions.

For qPCR, cDNA was generated using a High Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions (Invitrogen) from RNA isolated from five different HTM cell strains obtained from the eyes of the three donors. qPCR (7300 Real Time PCR System, Applied Biosciences) was used to evaluate mRNA levels using specific primers for GULP1, SIRPA, ABR, CD36, MFGE8, and CHN1 (Integrated DNA Technologies, Coralville, IA). The housekeeping gene SDHA was used as a reference gene. The results were normalized to untreated cells at day 0. The primers used for the qPCR reactions are listed in Table 1. Appendix 2 shows representative electropherograms of the RNA integrity used for qPCR. The RIN scores for this RNA were between 8.8 and 10.0 for both the DEX- and EtOH-treated samples. The 260/280 ratio was 2.04–2.11.

Transfections: TM-1 cells were grown on four-well chambered glass coverslips (ibidi USA, Madison, WI) at a density of 3.6×10^4 cells/ml for 24 h. Cells were then transfected with siRNA against human GULP1, SIRPA, ITGB5, or ABR using Mirus siQuest transfection reagent according to the manufacturer’s instructions (Mirus, Madison, WI) and as previously described [11,12]. The siRNA concentration was empirically determined for each gene, and qPCR was used to validate the knockdown of gene expression. A non-targeting (NT) siRNA (100 nM) and mock transfections were used as controls.

In some experiments, cells were transfected with the pcDNA3.1-CHN1-DYKDDDK plasmid using Mirus TransIT-LT1 transfection reagent (Mirus) as previously described [12]. DNA used in the transfections was obtained using a Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Inc., Valencia, CA).
**Phagocytosis of S. aureus bioparticles:** Forty-eight hours post-transfection, cells were serum starved in media containing 1% FBS for an additional 24 h before a phagocytic challenge. Transfected cells were then exposed to pHrodo™-conjugated S. aureus bioparticles for 4 h as previously described [12]. Cells were stained with CellMask Green (1/1000 dilution), and unfixed cells containing pHrodo™-labeled bioparticles were viewed using an inverted fluorescence microscope (Axio Observer, Zeiss, Thornwood, NY). Immunofluorescence acquisition software (Zen, Thornwood, NY) was used to capture the pHrodo™ fluorescence in green-labeled cells. Only cells that had engulfed at least three beads were counted as being phagocytic. The phagocytic activity was calculated by determining the ratio of cells containing pHrodo™-labeled bioparticles versus total number of cells from three to five separate randomly chosen areas per slide. Each experiment was independently repeated twice.

**Fluorescence-activated cell-sorting analysis:** Fluorescence-activated cell-sorting (FACS) analysis was performed as previously described [11]. Cells were suspended using cell dissociation buffer (Millipore Sigma, Burlington, MA). Cell suspensions were then incubated on ice for 1 h with mAbs to αvβ3 integrin (LM609), CD47, or CD36 at 5 µg/ml diluted in PBS with 2% BSA. Purified mouse IgG1 was used as a negative isotype control. Surface expression of CD47, CD36, and αvβ3 integrin was analyzed using the BD FACSCalibur System (BD Biosciences, San Jose, CA).

**Statistical analysis:** A National Eye Institute-funded University of Wisconsin Vision Core biostatistician was consulted for these analyses. Statistical analyses of the fold change in mRNA levels of the DEX- versus EtOH-treated HTM cells used a Mann–Whitney test, whereas qPCR analyses of the mRNA levels in three TM-1 cell lines were compared using a one-way ANOVA followed by a Tukey Honest Statistical Difference post-test. Statistical analyses for the phagocytosis assays used a Student t test. A p value <0.05 was considered significant.

**RESULTS**

**Gene microarray analysis:** Previous studies have reported that the treatment of HTM cells with DEX and/or the over-expression and activation of αvβ3 integrin by DEX [22,23] inhibited the phagocytic process in both normal HTM and immortalized TM-1 cells [11]. To understand how DEX could inhibit phagocytosis in HTM cells, we used gene microarray studies to look for proteins involved in phagocytosis whose expression of which was affected by DEX and hence might be involved in inhibiting phagocytosis in TM cells. Figure 1 shows the components we previously showed to be involved in TM phagocytosis [12]. We looked for changes in those proteins as well as other proteins that have been shown to be involved in phagocytosis in other cells [5,24,25]. The proteins we specifically looked for are shown in Figure 1. The changes included changes in the expression of various engulfment receptors, soluble eat-me signals, and adaptor molecules that help form the αvβ5 integrin/FAK complex involved in phagocytosis [5]. We also looked for GEFs or GTPase-activating proteins (GAPs) that could affect the role of RAC1 in phagocytosis in HTM cells.

The gene microarray analysis was performed on two separate HTM cell strains treated with 500 nM DEX or 0.1% EtOH vehicle for 5 d. As shown in Table 2, the gene microarray studies indicated that relatively few genes involved in the regulation of phagocytosis were affected by DEX. Only

**Table 1. Primers used for qPCR from the same 27 year old female donor.**

| Type      | Sequence                      |
|-----------|-------------------------------|
| Human GULP1 | f- TCCAATATCACACCAGTCTTCG     |
|           | r- CCACAGTTAAAATGGGTCAAAAGG   |
| Human SIRPA | f- TTCACTCTCAAGTCATCTCG       |
|           | r- ACATTCACCTGGTCTCTCG        |
| Human CD36  | f- GCCAGGTATTGCAGTTCTTTTC    |
|           | r- TGCTCGGGTTTTTCAACTGGAG    |
| Human MFG8  | f- GTACGTGAGATTGTAACCCAC      |
|           | r- ATCTGCTTGTCAAGGGATGC      |
| Human ABR   | f- CATCACCTTCTCTCTCTCG       |
|           | r- TGCTTGTCTCTCACTCCTCG      |
| Human CHN1  | f- TCAGGGAGTTGAATGTGCAAG      |
| (α2-chimaerin) | r- CGCTTAGTGTATGTGCTTTTC  |
| Human SDHA  | f- TGGGAACAAGGGGCATCTG       |
|           | r- CCACCACCTGCATCCAATCATG    |
seven genes were found to be altered in both DEX-treated cultures at a statistically significant level. Four genes (ITGβ3, CHN1, PIK3RI, and MFGE8) were upregulated by DEX, and three genes (ABR, GULP1, and CD36) were downregulated. With the exception of CD36 and MFGE8, which are an engulfment receptor and a soluble eat-me-signal, respectively, the other five genes are known to encode for proteins that can coordinate RAC1 activity.

The expression of four of the seven genes showed small changes of approximately two or threefold. However, three of the seven genes showed a greater than fourfold change in both cell strains. They were ITGB3 (β3 integrin subunit), CD36 (thrombospondin receptor), and PIK3RI (the PI-3 kinase regulatory subunit α1). Of the seven genes, all except CD36 and SIRPA were previously reported to be upregulated by DEX [19,21,23], and as shown in Table 2 the levels reported here closely match those found in an earlier microarray study by Rozsa et al. [21]. Except for αvβ3 integrin, the expression/activation of which was shown to have an inhibitory role in phagocytosis [11], none of the genes affected by DEX encoded for proteins previously reported to regulate phagocytosis in HTM cells [12]. These included αvβ5 integrin, RAC1, ELMO2, integrin-linked kinase (ILK), RHOG, and TIAM1 (Figure 1).
Table 2. DEX-induced changes in genes involved in phagocytosis.

| Gene Name | Protein Name | Function in Phagocytosis                                                                 | FC HTM1 | FC HTM2 | FC in Rozsa et al # (21) |
|-----------|--------------|------------------------------------------------------------------------------------------|---------|---------|--------------------------|
| CD36      | CD36 or Thrombospondin receptor            | class B scavenger receptor of pathogens, thrombospondin, collagen, lipoproteins, etc          | -10.2‡  | -5.3†   | NR                       |
| SIRPA     | Signal regulatory protein alpha or Tyrosine-protein phosphatase non-receptor type substrate 1 | Don’t eat me signal; binds CD47                                                               | -1.6*   | NC      | NR                       |
| ITGB3     | β3 integrin subunit                        | Binds ECM proteins; signals to regulate cytoskeleton                                           | 4.8‡    | 6.7‡    | 3.9                      |
| ABR       | Active breakpoint cluster region-related protein | Dual GEF/GAP Negative regulator of phagocytosis                                              | -2.3    | -1.6    | -1.4                     |
| CHN 1     | N-Chimaerin or α-chimaerin                  | GTPase activating protein (GAP) for Rac1                                                     | +3.0    | +3.0    | 1.5                      |
| GULP1     | PTB domain-containing engulfment adapter protein 1 | Regulates engulfment, coordinates Rac1 activity                                                | -2.9    | -3.6    | -2.5                     |
| PIK3R1    | Phosphoinositide-3-kinase regulatory subunit 1 | Phosphorylates phosphatidylinositol; involved in integrin, GPCR, RTK and MAPK signaling       | 4.1‡    | 4.3‡    | 2.8                      |
| MFGES     | Lactadherin or Milk fat globule-EGF factor 8 protein | Eat-me signal; binds β3 and β5 integrin                                                     | 2.0*    | 2.0‡    | 3.3                      |

Two separate HTM1 (N27TM-2) and HTM2 (N27TM-4) cell strains isolated from the same 27 year old female donor eye were used for this study. The fold change (FC) for HTM1 and HTM2 is the average of 5 and 3 biological replicates, respectively. Data for CD36, β3 integrin and PIK3R1 were previously published (19). Abbreviations: NR, not reported. NC, no change detected. * p< 0.05, † p < 0.01, ‡ p < 0.001, #only genes with a p value <0.005 were included.
To validate the observed changes in gene expression, we used qPCR and western blot analyses to evaluate changes at the mRNA and/or protein levels, respectively. In some instances, FACS analyses were performed to determine if any changes occurred at the protein level. Because the upregulation of mRNA for PIK3R1 and ITGB3 and their protein levels by DEX compared to EtOH-treated controls had previously been published [19,23], we did not pursue those studies here. However, as shown in Appendix 3 in the supplement, qPCR analyses supported our previous studies [19,23] and indicated that mRNA levels for ITGB3 and PIK3R1 were also upregulated in the HTM cell strains used in this study.

**DEX affects the expression of GAPs that regulate RAC1 activity:** The first genes examined were CHN1 and ABR (Table 2). As shown in Figure 2A, CHN1 encodes for a protein called α-chimaerin that can be alternatively spliced into two isoforms called α1-chimaerin and α2-chimaerin [26]. Both forms contain a GAP domain that specifically targets the activity of RAC1 (Figure 1) and therefore would be considered a negative regulator of RAC1-mediated phagocytosis in TM cells [12]. α2-chimaerin differs from α1-chimaerin in that it contains an SH2 domain and a GAP domain; thus, it can act as both a GAP protein and an adaptor protein, whereas α1-chimaerin can only act as a GAP protein.

To determine which form of α-chimaerin is found in HTM cells, we first performed PCR using primers specific for each isoform [27]. Brain tissue that should only contain the α1-chimaerin variant was used to demonstrate the specificity of the primers [28]. As shown in Figure 2A, brain tissue expressed α1-chimaerin but not α2-chimaerin. In contrast, only α2-chimaerin was detected in HTM and TM-1 cells, indicating that this was the isoform found in TM cells (Figure 2A) and that α2-chimaerin could have multiple functions in the TM besides regulating RAC1 activity.

qPCR was then performed to determine if mRNA levels of the α2-chimaerin splice variant were upregulated following DEX treatment. As shown in Figure 2B, qPCR was then performed to determine if mRNA isolated from HTM cells supported the microarray studies and showed a two to threefold increase in the mRNA levels in cultures at days 2, 4, and 5 of DEX treatment compared to untreated (no trt) cultures. However, only the increase at day 2 appeared to be significant (*, p<0.05). In contrast, the mRNA levels in EtOH-treated control cultures remained unchanged compared to the no trt cultures. Western blot analysis of cell lysates isolated from HTM cell cultures supported the microarray data and showed an increase in α-chimaerin protein levels in HTM cells after 5 d of treatment with DEX compared to cultures treated with the EtOH vehicle (Figure 2C).

To see if α2-chimaerin played a role in phagocytosis, we overexpressed full-length FLAG-tagged α2-chimaerin in TM-1 cells and looked for any change in their phagocytic activity. A mock transfection without the presence of DNA was used as a control to detect any potential side effects of the transfection reagent. The expression of FLAG-tagged α2-chimaerin could be detected along the edge of the cells 48 h after transfection (Appendix 4). Despite the increase in α2-chimaerin expression, TM-1 cells transfected with FLAG-tagged α2-chimaerin did not show any significant change in their phagocytic capabilities compared to mock transfected cells (Figures 2D-E). This suggests that although DEX may alter the expression of α2-chimaerin, α2-chimaerin does not appear to have a role in phagocytosis in TM cells.

The next gene we looked at was ABR. ABR encodes for the active BCR-related protein [29-31]. It contains both a GEF domain that can activate RHO GTPases and a GAP domain that has been shown to specifically inhibit RAC1 activity (Figure 3A) during phagocytosis in macrophages [29]. Therefore, as shown in Figure 1, we placed it as a potential negative regulator of RAC1 activity during phagocytosis in TM cells.

qPCR analysis of ABR mRNA levels in HTM cells showed the levels were significantly decreased (*, p<0.05) in DEX-treated cultures on day 5 compared to untreated cultures (Figure 3B). However, a decrease in ABR mRNA levels was also observed in the EtOH-treated control cultures. Although this decrease was not statistically significant, it did raise concerns that the decrease in ABR levels at day 5 in DEX-treated cells may not be due to the DEX treatment but rather to the vehicle. The increases in ABR mRNA levels after 2 and 4 d of EtOH were also not statistically significant compared to the untreated cultures. Western blot analyses of HTM cell lysates failed to show a significant change in ABR protein levels (Figure 3C) in DEX-treated cells compared to EtOH-treated cells, further supporting the idea that DEX did not affect ABR levels. Why there was a change in ABR’s mRNA level and not its protein level is unclear. One possible explanation is that the half-life of ABR mRNA and its protein in cultured HTM cells differ.

Nevertheless, we did examine the effect of knocking down ABR mRNA levels on phagocytosis. Using siRNA specific to ABR, we obtained a 59% knockdown in ABR mRNA levels by qPCR compared to the NT siRNA control (data not shown). Interestingly, when siRNAs were used to knock down mRNA levels of ABR in TM-1 cells, we did see an effect on phagocytosis (Figures 3D, E). As shown in Figure 3E, the knockdown of ABR mRNA resulted in a statistically significant decrease (*; p<0.05; 36%) in phagocytosis compared to the control cells transfected with NT siRNAs.
This suggests that even though we could not demonstrate a change in ABR protein levels by day 5 of DEX treatment, ABR does appear to be involved in phagocytosis.

DEX treatment reduces GULP1 expression and the knockdown of GULP1 inhibits phagocytosis: Another gene that was found to be altered by DEX was GULP1. GULP1 is an adaptor...
protein that coordinates engulfment signaling pathways between the αvβ5 integrin and its co-receptor, stabilin-2, at sites of phagocytosis in erythrocytes [32]. Therefore, we placed it interacting with αvβ5 integrin in the pathway shown in Figure 1. As shown in Figure 4A, mRNA levels for GULPI isolated from HTM cells were significantly (p<0.05,*) downregulated in HTM cells treated with DEX at days 2, 4, and 5 compared to the no trt control and compared to EtOH-treated cells. A corresponding decrease in protein expression was also observed upon western blot analysis of cell lysates from Figure 3. Expression of the dual GEF/GAP ABR is unaffected by DEX, but it plays a role in phagocytosis. A: Schematic diagram showing that ABR contains both a GEF and a GAP domain. It addition, it contains a pleckstrin homology (PH) domain and a C1 domain that mediates binding to diacylglycerol and phorbol esters. B: qPCR analyses showed that 5 d of DEX treatment caused the downregulation of mRNA for ABR compared to the no trt group. The decrease at day 5 was statistically significant (*, p<0.05) compared to the no trt group but not compared to EtOH-treated cells at day 5. The mRNA levels were normalized to the no trt group. Data are presented as the mean ± SEM. All five HTM cell strains (N27TM-2, N27TM-4, N27TM-5, N27TM-6, and N25TM-8) were used for the qPCR analyses; n=5. C: Western blot analyses showed that protein levels of ABR were unaffected by DEX compared to EtOH-treated controls. β-actin was used as a loading control. Equal amounts of protein from the DEX- and EtOH-treated cell lysates were loaded. The figure is a representative blot done on cell lysates from three separate experiments using the HTM cell strain (N25TM-8). n=3. D: Fluorescence micrographs showed a marked decrease in the uptake of pHrodo™ bioparticles (red) in TM-1 cells transfected with ABR siRNA compared to NT siRNA transfected cells. Cells were counterstained with CellMask Green to visualize the cells. E: Quantification of the number of TM-1 cells that took up pHrodo™ bioparticles. Cultures transfected with siRNA to ABR showed a statistically significant (*p<0.05) reduction in phagocytosis (n=3580 cells counted) compared to cells transfected with NT siRNA (n=1460 cells counted). Data are presented as the percent positive ± SEM.
HTM cells (Figure 4B), thus supporting the change in gene expression detected by the microarray analysis (Table 2). To see if reduced levels of GULPI affected phagocytosis, we used siRNAs to knock down its expression in TM-1 cells before the phagocytosis assay. qPCR analysis showed that GULPI siRNA produced a 46% knockdown in GULPI mRNA levels compared to the NT siRNA control (data not shown). The ITGB5 siRNA produced a 96% knockdown in ITGB5 mRNA levels compared to the NT siRNA control (data not shown), which supported our previous results [11]. As shown in Figure 4C–D, the knockdown of GULPI in TM-1 cells resulted in a statistically significant (p<0.05) decrease in phagocytosis by 40%. This is similar to the decrease in phagocytosis observed when ITGB5 expression, which we previously showed played a role in phagocytosis [11], was knocked down (Figure 4C-D). In contrast, the NT siRNA had no effect on phagocytosis.

_DEX reduces expression of SIRPA but not its ligand CD47: We then investigated the role of SIRPA. As shown in Table 2, one of the HTM cell strains in our microarray study indicated that DEX treatment significantly decreased the expression of SIRPA by 1.6 fold. SIRPa is a transmembrane tyrosine phosphatase and is best known as a “don’t eat-me-signal” during phagocytosis as a way to protect healthy cells from being engulfed [33]. This protective mechanism is triggered when SIRPa binds its ligand CD47 on the phagocyte, as shown in Figure 5A. However, both SIRPa and CD47 also play a role in regulating αvβ3 integrin signaling [34], which inhibited phagocytosis in TM cells. SIRPa (also known as SHPS1) can be found in focal adhesions with integrins, and it negatively regulates β3 integrin-mediated adhesion in platelets [35]. CD47 (also known as integrin associated protein or IAP) is a co-receptor that mediates the activity of integrins [36,37] (Figure 5A) and also appears to be involved in activating αvβ3 integrin signaling in HTM cells [38]. Therefore, both SIRPa and CD47 could also be involved in the αvβ3 integrin signaling pathway in TM cells that inhibited phagocytosis in TM cells [11].

As shown in Figure 5B, qPCR analyses indicated that mRNA levels of SIRPA in HTM cells were significantly decrease (*, p<0.05) by day 5 of DEX treatment compared to untreated cells. In contrast, EtOH-treated controls showed a significant increase in mRNA levels in SIRPA at days 2 and 4 compared to the no trt controls. At day 5, however, SIRPA mRNA levels in EtOH-treated controls returned to the baseline levels observed in no trt HTM cells. Western blot analyses also verified the microarray study and showed a decrease in protein levels of SIRPa in HTM cell lysates (Figure 5C). Unfortunately, attempts to examine the role of SIRPa in phagocytosis were not possible, as the knockdown of SIRPA expression using siRNAs caused the cells to detach from the plates, suggesting it had a role in cell adhesion in TM cells [35].

Because it is possible that CD47 protein levels could have been affected in DEX-treated HTM cells, even though gene expression was not, we used FACS analysis to examine the levels of CD47 on the cell surface of HTM cells treated with DEX. As shown in Figure 5D, CD47 expression was unaffected in DEX-treated HTM cells relative to control cells treated with EtOH, which supports the observation that we did not see any change in our microarray studies. To ensure that the cells were responding to DEX, we also looked at the levels of αvβ3 integrin. FACS analysis indicated the expression of αvβ3 integrin was upregulated by DEX, which demonstrates that the cells did respond to DEX. It also supports the microarray data in this study (Table 2) and in our previous studies [19,22,23], namely that DEX affects αvβ3 integrin expression. This suggests that in DEX-treated cells CD47 may be free on the cell surface to participate in interactions with αvβ3 integrin (Figure 5A) if SIRPa levels were downregulated.

_Overexpression of β3 integrin subunit affects GULPI and ABR expression:_ Because previous studies [11] showed that overexpression of a constitutively active CA αvβ3 integrin results in a statistically significant decrease in phagocytosis in immortalized TM cells, we asked if this correlated with any of the changes we observed in the DEX-treated HTM cells. Using qPCR, we examined the levels of GULPI, CHNI, ABR, and SIRPA mRNAs in TM-1 cells overexpressing WT or CA αvβ3 integrin compared to control TM-1 cells transfected with an EV [11]. Figure 6A shows that overexpression of both WT and CA αvβ3 integrin caused a statistically significant decrease in GULPI mRNA levels compared to control cells. Interestingly, overexpression of WT or CA αvβ3 integrin had the opposite effect of DEX on CHNI mRNA levels and caused a decrease in the mRNA level compared to control cells (Figure 6B). SIRPA mRNA levels, however, were unaffected by the overexpression of either the WT or CA αvβ3 integrin (Figure 6C). The overexpression of WT αvβ3 integrin also caused a slight but statistically significant decrease in ABR mRNA levels compared to control cells, while ABR mRNA levels were unaffected in cells expressing CA αvβ3 integrin (Figure 6D). This suggests that αvβ3 integrin expression affects the transcription of proteins that regulate RAC1 activity in TM-1 cells. However, only GULPI mRNA expression appears to correlate with the downregulation of phagocytosis observed in response to DEX treatment and αvβ3 integrin expression.
Figure 4. Knock down of GULP1 expression inhibits phagocytosis. A: qPCR analyses showed that DEX treatment caused the downregulation of GULP1 mRNA by day 2 of treatment compared to the no trt and the EtOH-treated controls. The decrease on all three days was statistically significant compared to the no trt cells and EtOH-treated cells (*p<0.05). Data are presented as the mean ± SEM. The mRNA levels were normalized to the no trt group. All five HTM cell strains (N27TM-2, N27TM-4, N27TM-5, N27TM-6, and N25TM-8) were used for the qPCR analyses; n=5. B: Western blot analyses showed that protein levels of GULP1 were decreased in DEX-treated cells compared to EtOH-treated controls. The figure is a representative blot done on HTM (N25TM-8) cell lysates from three separate experiments; n=3. β-actin was used as a loading control. Equal amounts of protein from the DEX- and EtOH-treated cell lysates were loaded. C: Fluorescence micrographs showed a decrease in the uptake of pHrodo™ bioparticles (red) in TM-1 cells transfected with GULP1 and ITGB5 siRNA compared to NT siRNA-transfected cells. Cells were counterstained with CellMask Green to visualize the cells. White arrows point out that many cells transfected with GULP1 or ITGB5 siRNAs have engulfed fewer bioparticles/per cell than NT siRNA-transfected cells. D: Quantification of the number of cells that took up pHrodo™ bioparticles in TM-1 cells transfected with GULP1 siRNA (n=1610 cells) or ITGB5 siRNA (n=1605 cells) showed a statistically significant (*) reduction in phagocytosis at p<0.05 compared to cultures transfected with NT siRNA (n=1888 cells). Data are the percent positive ± SEM of three independent experiments.
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**Figure 5. Effect of DEX on CD47 and SIRPα expression.** A: Schematic diagram showing that CD47 can interact with αvβ3 integrin on the same cell or with SIRPα on an adjacent cell. CD47 can also bind thrombospondin-1 (TSP), which promotes an interaction between CD47 and αvβ3 integrin and triggers αvβ3 integrin signaling [36,37]. B: qPCR analyses showed that DEX treatment caused the downregulation of SIRPα mRNA by day 5 of treatment compared to the no trt group at day 0. In contrast, EtOH-treated controls showed an increase in mRNA levels starting on day 2 of the treatment. All these changes were statistically significant (*) at p<0.05 compared to the no trt cells. The mRNA levels were normalized to the no trt group. All five HTM cell strains (N27TM-2, N27TM-4, N27TM-5, N27TM-6, and N25TM-8) were used for the qPCR analyses; n=5. C: Western blot analyses showed that protein levels of SIRPα were decreased by DEX compared to EtOH-treated controls. The figure is a representative blot done on cell lysates from three separate experiments using the N25TM-8 cell strain. β-actin was used as a loading control. Equal amounts of protein from the DEX- and EtOH-treated cell lysates were loaded; n=3. D: FACS analyses of CD47 and αvβ3 integrin levels in HTM cells (N27TM-4) in the presence of DEX or a vehicle. Analyses were done in duplicate and verified in two independent experiments using both N27TM-2 and N27TM-4 cells. DEX caused an increase in αvβ3 integrin levels but not CD47; n=2.

**DEX affects MFG-E8 but not CD36 mRNA levels:** Finally, we looked at the expression of CD36 and MFG-E8. CD36 (also known as the thrombospondin receptor) is a scavenger receptor for matrix proteins, apoptotic cells, and lipoproteins during integrin-mediated phagocytosis [39,40]. MFG-E8 is the milk factor globule-EGF factor 8, also known as lactadherin. MFG-E8 is a secreted protein that functions as a bridging protein in phagocytosis. It binds to apoptotic cells and then via interactions with αvβ3 and αvβ5 integrins on the phagocytes targets the apoptotic cells for phagocytosis. Although it predominantly functions as an “eat-me signal” of apoptotic cells, MFG-E8 can also mediate the removal of collagen debris during phagocytosis [41]. qPCR analysis of MFG-E8 mRNA levels in HTM cells verified the microarray data (Table 2) and showed that MFG-E8 mRNA was upregulated after 2, 4, and 5 d of DEX treatment of HTM cells compared to the EtOH-treated cells (Figure 7A). Because western blot analyses failed to detect MFG-E8 in cell lysates, changes at the protein level could not be verified.

With regard to CD36, the large decrease in mRNA levels predicted by the microarray data could not be verified by qPCR analyses (Figure 7B). In fact, we saw large variations in the expression of CD36 mRNA in DEX-treated cells between the different HTM cell strains. Of the five cell strains examined, three HTM cell strains from the same donor showed a decrease in CD36 expression, which supported the microarray data based on RNA obtained from those same HTM cell strains. In contrast, one strain from a different donor showed an increase in mRNA levels, while another HTM cell strain from the third donor showed no change. We saw a similar effect at the protein level using FACS to detect CD36 levels on the cell surface. In this instance, we saw CD36 levels downregulated in one HTM cell strain in response to DEX but not in another cell strain, despite the fact that both cell strains were from the same donor. When we averaged the levels of CD36 expression in the different cell strains, we saw no statistical difference in DEX-treated HTM cells compared to the EtOH-treated controls (Figure 7C).
Figure 6. Effect of αvβ3 integrin expression on GULP1, CHN1, ABR, and SIRPA mRNA expression. qPCR analyses of GULP1, CHN1, SIRPA, and ABR mRNA levels were done on stable TM-1 cell lines overexpressing the WT β3 integrin subunit or a CA β3 integrin subunit [11]. A cell line transduced with an empty vector (EV) was used as a control. Analyses were done in triplicate in three independent experiments; n=3. The decreases in GULP1, CHN1, and ABR mRNA levels were statistically significant (*) at p<0.05 compared to the cells transduced with the EV. All data were normalized to the mRNA levels observed in EV cells. Data represent the mean ± SEM.

Figure 7. Effect of DEX on MFG-E8 and CD36 expression. A: qPCR analyses showed that DEX treatment increased the expression of MFG-E8 mRNA at days 2, 4, and 5 compared to the no trt controls. The increases were statistically significant (*) at p<0.05) compared to the no trt controls. The mRNA levels were normalized to the no trt group. All five HTM cell strains (N27TM-2, N27TM-4, N27TM-5, N27TM-6, and N25TM-8) were used for the qPCR analyses; n=5. B: qPCR indicated that DEX did not have any statistically significant effect on mRNA levels for CD36 compared to the no trt HTM cells at day 0. The mRNA levels were normalized to the no trt group. All five HTM cell strains described in (A) were used for the qPCR analyses; n=5. C: FACS analyses of CD36 levels on HTM cells (N25TM-8 and N25TM-10) in the presence of DEX or a vehicle were done in duplicate. Cell surface CD36 levels did not change significantly in response to DEX; n=2. All data represent the mean ± SEM.
DISCUSSION

Phagocytosis plays an essential role in outflow facility by clearing the outflow pathway of cellular debris and mediating tissue homeostasis. Following treatments with glucocorticoids, this process is impaired in TM cells. In this study, we showed that DEX treatment affects several genes known to be involved in phagocytosis [5] but until now have never been identified to be part of the engulfment pathway in TM cells. Interestingly, all the genes affected were either associated with modulating RAC1 activity (ABR and CHN1) or involved in integrin signaling (CD36, GULP1, ITGB3, PIK3R1, and MFGE8). This suggests that the major effect of DEX and αvβ3 integrin on phagocytosis may be an alteration in the integrin-mediated RAC1 pathway involved in phagocytosis. Previous studies suggested that the overexpression of an activated αvβ3 integrin by DEX may be responsible for the decrease in αvβ5 integrin-mediated phagocytosis in TM cells [11,12]. How αvβ3 integrin signaling inhibits phagocytosis is not known. Here, we extended our previous studies and compared changes in the phagocytic pathway induced by DEX to those observed during the overexpression of αvβ3 integrin. These studies show that DEX and αvβ3 integrin signaling affected the expression of several genes involved in phagocytosis. One gene they both downregulated was GULP1. This suggests that DEX and αvβ3 integrins may inhibit phagocytosis in part by changing the transcription of genes needed for phagocytosis and that those changes are triggered by the DEX-induced upregulation of αvβ3 integrin expression [23].

GULP1 is an adaptor protein that binds the engulfment receptor stabilin-2 and helps coordinate the interactions between the various independent signaling pathways involved in forming the phagocytic cup. In particular, it coordinates signaling events between αvβ5 integrins and stabilin-2 [32,42,43]. Because previous studies have shown that phagocytosis in TM cells involves αvβ5 integrins, it is likely that GULP1 plays a similar role in TM cells. Whether stabilin-2 is also involved in TM cell phagocytosis is not clear, as we have not been able to show stabilin-2 expression in our HTM cells.

Drawing analogies from other studies, we propose that GULP1 may help to coordinate the ILK/RHOG/ELMO2 and TIAM1 pathways shown in Figure 1 that activate RAC1 in TM cells during phagocytosis [12]. We also suggest that both DEX and αvβ3 integrin inhibit phagocytosis by downregulating GULP1 expression that would then disrupt the coordinated signaling between ILK/RHOG/ELMO2 and TIAM1 upstream of RAC1 [12]. These studies therefore suggest that the DEX-induced inhibition of phagocytosis is caused by the upregulation of αvβ3 integrin expression by DEX [23], which in turn downregulates the expression of GULP1.

GULP1 is probably not the only factor responsible for inhibiting phagocytosis in DEX-treated TM cells, as the knockdown of GULP1 only inhibited phagocytosis by 40%. This partial inhibition has been seen in other phagocytes [32] and indicates that another GULP1-independent engulfment pathway is involved and still active when GULP1 is knocked down. One potential candidate is an ABR-mediated pathway. Although its expression was not altered by DEX or the activation of αvβ3 integrin signaling, the knockdown of ABR expression did result in a partial inhibition (36%) of phagocytosis. This further supports the idea that there are multiple pathways involved in regulating phagocytosis, some of which may not be affected by DEX and αvβ3 integrin signaling.

How ABR can affect phagocytosis is an interesting question. ABR is a dual GEF/GAP protein that specifically inhibits RAC1 activity through its GAP domain by interacting with TIAM1 [31]. In contrast, the GEF domain of ABR activates several GTPases, including RHOA, RAC1, and CDC42 [44]. Because the knockdown of ABR’s GAP activity would enhance RAC1 activity and phagocytosis [29], the inhibition of TM phagocytosis following the knockdown of ABR implies that it is the function of the GEF domain and its downstream target that is being impacted. The downstream target of ABR is unknown. However, recent studies have shown that a GEF is needed to activate the ELMO1/RHOG/DOCK1 complex involved in phagocytosis [45]. While the ELMO1/RHOG/DOCK1 complex is not involved in TM phagocytosis, an ILK/RHOG/ELMO2 complex is [12]. Therefore, we suggest that ABR could function as a GEF to activate the ILK/RHOG/ELMO2 complex involved in TM phagocytosis (Figure 1). To date, however, no role for ABR as a GEF for this complex has been shown.

Other than CD36, our studies did not find any evidence that DEX affects the expression of engulfment receptors [42]. It is unlikely that CD36 plays a major role in TM phagocytosis, as our qPCR and FACS analyses have shown that the expression of CD36 varies among the different cell strains and donors. Furthermore, TM-1 cells do not express CD36 on their cell surface (data not shown) and yet they are phagocytic [12]. This agrees with other published research that has yet to clarify the importance of CD36 in phagocytosis [46].

It is still unclear what role, if any, SIRPa and CD47 play in phagocytosis. SIRPa levels were clearly downregulated by DEX, while CD47 expression was not affected. This would leave CD47 free to activate the αvβ3 integrin pathway that inhibited phagocytosis [11,36]. Prior studies showing that CD47 can cause the activation of αvβ3 integrin in TM cells
support this idea [38]. In those studies, the activation of αvβ3 integrin resulted in the formation of cross-linked actin networks or CLANs [22,38,47], which is another phenotypic change associated with DEX treatment and glaucoma in TM cells [48,49]. Therefore, although SIRPα may not be directly involved in phagocytosis, changes in its expression suggest a way that the activity of αvβ3 integrin can be regulated in DEX-treated cells.

Interestingly, we saw an increase in MFGE8 expression, which is considered to be an “eat-me signal.” MFG-E8 functions as a bridge protein between phosphatidylserine on apoptotic cells and αvβ5 integrin on phagocytes to mediate the clearance of apoptotic debris [43]. It has also been shown to mediate the removal of excess collagen in fibrotic lungs [50,51] and thus could also play a role in preventing the excess deposition of extracellular matrix proteins upregulated by DEX in TM cells [52,53] in an attempt to maintain tissue homeostasis. This would be consistent with the role MFG-E8 plays in promoting tissue regeneration in intestinal epithelia [54] or interacting with αvβ3 and αvβ5 integrin to induce vascular endothelial growth factor-dependent survival signals in angiogenesis [55]. Therefore, its upregulation could have a tissue-protective function. In fact, because it is also a ligand for αvβ3 integrin, MFG-E8 has the potential ability to activate the αvβ3 integrin signaling pathway that downregulates phagocytosis. Whether the potential activation of αvβ3 integrin signaling by MFG-E8 or CD47 is part of a homeostatic feedback loop to control phagocytosis and protect the TM remains to be determined.

In summary, the major effect of DEX on phagocytosis in TM cells appears to be a disruption of the αvβ5 integrin/RAC1-mediated engulfment pathway and not the expression of cell surface engulfment receptors. The study also suggests there are at least two independent engulfment pathways that are important in TM cell phagocytosis, and we have identified several new proteins involved in the phagocytic pathway in TM cells. Finally, the study suggests that αvβ3 integrin signaling may participate in the coordination of these pathways by transcriptionally regulating proteins responsible for the downregulation of phagocytosis.

APPENDIX 1. STR ANALYSIS.
To access the data, click or select the words “Appendix 1.”

APPENDIX 2. ASSESSMENT OF RNA INTEGRITY.
To access the data, click or select the words “Appendix 2.” Panels A-F show representative profiles of the 18S and 28S ribosomal RNA subunits from 3 of the 5 cell strains used in this study. Cells were treated with either DEX (A, C, and E) or EtOH vehicle for 5 days. RNA was isolated as described in methods. The RIN score for the 6 samples was between 8-10. Panels A and B show representative electropherograms of the RNA used in the gene microarrays whereas panels C-F show representative electropherograms of the RNA used for the qPCR experiments.

APPENDIX 3. DEX INCREASES THE EXPRESSION ITGB3 AND PI3KR1 IN HTM CELLS.
To access the data, click or select the words “Appendix 3.” qPCR analyses confirmed prior studies and showed that expression of ITGB3 and PI3KR1 were upregulated by DEX by Day 5. Analyses were done on RNA isolated from 5 separate HTM cell strains (N27TM-2, N27TM-4, N27TM-5, N27TM-6, and N25TM-8). The increases were statistically significant (*) at p<0.05 relative to EtOH treated levels. Data are presented as the mean ± S.E.

APPENDIX 4. IMMUNOFLUORESCENCE MICROSCOPY OF FLAG TAGGED-α2-CHIMAERIN EXPRESSION.
To access the data, click or select the words “Appendix 4.” FLAG tagged-α2-chimaerin (arrows) was only detected along the edges of the cell membranes in TM-1 cells transfected with the pcDNA3.1-CHN1-DYKDDDDK plasmid and not in the mock transfected cells (no plasmid added). In contrast, only diffuse staining was detected in cultures labeled with IgG control. Scale bar=100µm. Forty-eight hrs post-transfection, TM-1 cultures were processed for immunolabeling as we previously described (22). Cultures were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Fixed cells were blocked with 1% BSA and then stained with a 1/100 dilution of either a IgG isotype control or anti-FLAG IgG (F1804, Sigma) for 1 hr. Primary antibodies were detected with Alexa 488-conjugated goat anti-mouse IgG diluted 1/500 for 30 min.

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